NATIONWIDE DISTRIBUTION AND INSECTICIDE RESISTANCE STUDY OF MALAYSIAN MOSQUITO *CULEX QUINQUEFASCIATUS* SAY BY MOLECULAR AND BIOCHEMICAL TOOLS

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ABSTRACT

A nationwide investigation was carried out to (1) determine the distribution of *Culex quinquefasciatus* and other species of mosquitoes in stagnant water in residential areas, (2) investigate the genetic diversity of *Cx. quinquefasciatus*, (3) quantify the insecticide susceptibility status of *Cx. quinquefasciatus*, (4) characterize the biochemical mechanisms of insecticide resistance in *Cx. quinquefasciatus*, (5) characterize the molecular mechanisms of insecticide resistance in *Cx. quinquefasciatus* from 13 states and one federal territory in Malaysia. *Culex* larval surveillance indicated that *Cx. quinquefasciatus* was the predominant species in residential areas. Several habitat characteristics (i.e., pH, conductivity, salinity, total dissolved solids, elevation and dissolved oxygen) were found to be associated with *Culex* larvae distribution. In the context of molecular phylogeography, the genetic diversity of Malaysian *Cx. quinquefasciatus* was extremely low since only three and four haplotypes were revealed by COI and COII, respectively. As for insecticide resistance study of *Cx. quinquefasciatus*, both WHO larval and adult bioassays exhibited dissimilar trends in susceptibility against DDT, propoxur, malathion and permethrin. Correlations between propoxur and malathion resistance as well as between propoxur and permethrin resistance in larval bioassays were found. In enzyme microassays, elevated levels of α-esterases, β-esterases, mixed function oxidases, glutathione-S-transferase and acetylcholinesterase activities were demonstrated in majority of the populations. Besides, a correlation between α-esterases activity and malathion resistance was also demonstrated. An association between activity of α-esterases and β-esterases and between glutathione-S-transferase and acetylcholinesterase was also demonstrated. In addition, genotyping of insensitive acetylcholinesterase revealed the presence of G119S mutation in the wild populations of *Cx. quinquefasciatus*, but in heterozygous state and

at a very low frequency (18 out of 140). Statistical analysis also revealed that malathion resistance was associated with the frequency of *ace-1* resistant allele in *Cx. quinquefasciatus* populations. On the other hand, genotyping of insensitive voltage gated sodium channel revealed the presence of L1014F mutation which was dominated by heterozygous genotype (99 out of 140), followed by three individuals of homozygous genotype. However, no association was found between the frequency of *kdr* resistant allele and DDT and permethrin resistance. The findings of this study could be utilized in the implementation of strategic measures in vector control programs in Malaysia. Identification of the breeding preferences and population structure of mosquitoes offers an opportunity to elucidate their influence on the current distribution of mosquito species in these study areas and subsequently understand their potential risks in disease transmission. Besides, the bioassay data may also assist local authorities in providing susceptibility baseline and appropriate dosage of larvicide or adulticide to be used during vector control operations. Application of both biochemical and molecular tools provides significant insights into the evolution and adaptation of Malaysian *Cx. quinquefasciatus*. In conclusion, this study has demonstrated the associations between the *Culex* distribution and various habitat characteristics. Besides, a phylogenetic relationship among Malaysian *Cx. quinquefasciatus* was revealed. This study has provided the first evidence for the involvement of target site alterations and detoxification mechanisms in insecticide resistance among *Cx. quinquefasciatus* populations in Malaysia.

ABSTRAK

Satu kajian di seluruh negara telah dijalankan untuk (1) menentukan taburan nyamuk *Culex quinquefasciatus* dan spesis nyamuk lain dalam air bertakung di kawasan perumahan, (2) meninjau kepelbagaian genetik *Cx. quinquefasciatus*, (3) menentukan tahap kerentanan *Cx. quinquefasciatus* terhadap racun serangga, (4) mengenal pasti mekanisme biokimia kerintangan racun serangga dalam *Cx. quinquefasciatus*, (5) mengenal pasti mekanisme molekul kerintangan racun serangga dalam *Cx. quinquefasciatus* dari 13 negeri dan Wilayah Persekutuan di Malaysia. Peninjauan yang dilakukan terhadap larva *Culex* menunjukkan bahawa *Cx. quinquefasciatus* merupakan species larva yang utama di kawasan perumahan. Beberapa ciri habitat (pH, kekonduksian, kemasinan, jumlah pepejal terlarut, ketinggian tapak dari aras laut dan oksigen terlarut) didapati berkait dengan taburan larva *Culex*. Dalam aspek filogeografi molekul, tahap kepelbagaian genetik *Cx. quinquefasciatus* di Malaysia adalah sangat rendah disebabkan terdapat hanya tiga dan empat haplotip yang dikesan dengan gen COI dan COII. Dalam kajian kerintangan *Cx. quinquefasciatus* terhadap racun serangga, WHO bioassai pada peringkat larva dan dewasa menunjukkan tahap kerentanan yang berbeza terhadap DDT, propoxur, malathion dan permethrin. Hubungan antara kerintangan propoxur dengan malathion dan propoxur dengan permethrin telah menunjukkan kesan melalui bioassai larva. Dalam kajian mikroassai enzim, peningkatan tahap aktiviti-aktiviti α-esterase, β-esterase, oxidase fungsi campuran, glutation-S-transferase dan asetilkolinesterase telah didapati di kebanyakan populasi. Selain itu, terdapat juga hubungan di antara aktiviti α-esterase dengan kerintangan malathion. Hubung kait di antara aktiviti α-esterase dan β-esterase dan antara glutation-S-transferase dan acetylcholinesterase juga dikenalpasti. Disamping itu, ujian pengesanan alel *ace-1* menunjukkan terdapat kehadiran mutasi G119S di populasi *Cx.*

quinquefasciatus yang diperolehi dari lapangan tetapi dalam keadaan heterozigot dan berada dalam frekuensi yang rendah (18 daripada 140). Analisis statistik menunjukkan bahawa kerintangan malathion berkait rapat dengan frekuensi alel *ace-1* dalam populasi *Cx. quinquefasciatus*. Selain itu, ujian pengesanan alel *kdr* juga menunjukkan kewujudan mutasi L1014F yang didominankan oleh genotip heterozigot (99 daripada 140), diikuti dengan tiga individu genotip homozigot. Walau bagaimanapun, tiada hubung kait ditunjukkan antara frekuensi alel *kdr* dengan kerintangan DDT dan permethrin. Hasil daripada kajian ini dapat digunakan dalam melaksanakan langkah-langkah strategik bagi program kawalan vektor di Malaysia. Pengenalpastian kawasan pembiakan yang sesuai dan struktur populasi nyamuk dapat menjelaskan pengaruh nyamuk ke atas taburan spesiesnya di kawasan-kawasan kajian dan memahami potensi risiko terhadap jangkitan penyakit. Selain itu, data bioassai juga dapat membantu pihak berkuasa tempatan dalam menyediakan maklumat asas mengenai kerentanan dan dos racun serangga yang sesuai semasa operasi kawalan vektor. Penggunaan teknik-teknik biokimia dan molekul dapat mempertingkatkan lagi pengetahuan dalam aspek evolusi dan adaptasi *Cx. quinquefasciatus* di Malaysia. Kesimpulannya, kajian ini telah menunjukkan hubung kait antara taburan nyamuk *Culex* dan ciri-ciri habitatnya. Selain itu, kajian ini juga telah menunjukkan hubungan filogenetik *Cx. quinquefasciatus* dan membuktikan kewujudan perubahan tapak target serta tahap aktiviti enzim dalam kerintangan racun serangga oleh *Cx. quinquefasciatus* di Malaysia.

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&	and
^{O}	degree
=	equal to
≤	less than or equal to
<	less than
>	greater than
^{o}C	degree Celsius
%	percent
et al.	et alia (“and others”)
i.e.	id est (“that is”)
WHO	World Health Organization
DNA	deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
mRNA	messenger ribonucleic acid
tRNA	transfer ribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid
ATP	adenosine triphosphate
Cx.	*Culex*
Ae.	*Aedes*
An.	*Anopheles*
Ma.	*Mansonia*
Lu.	*Lutzia*
Ar.	*Armigeres*
ULV	ultra low volume
DDT	dichlorodiphenyltrichloroethane or 1,1,1 -trichloro-2,2-bis (chlorophenyl) ethane
EST	esterases
MFO	mixed function oxidases
GST	glutathione-s-transferases
GSH	glutathione
AcHE	insensitive acetylcholinesterase
KD	knockdown
kdr	knockdown resistance
GABA	gamma aminobutyric acid
LC_{50}	50% lethal concentration
KT_{50}	50% knockdown time
RR	resistance ratio; homozygous resistance
RS	heterozygous susceptible
SS	homozygous susceptible
R	resistant
S	susceptible
M	moderate resistant
PAGE	polyacrylamide gel electrophoresis
2D-electrophoresis	two-dimensional electrophoresis
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphism
AS-PCR	allele-specific-polymerase chain reaction
HOLO	hot ligation oligonucleotide assay

SSOP	sequence-specific oligonucleotide probes
FRET/MCA	fluorescence resonance energy transfer/melt curve analysis
pH	potential of hydrogen
TDS	total dissolved solids
DO	dissolved oxygen
DI	dipper index
BI	breeding index; bayesian inference
TLP	total number of larvae
ND	number of dips
BP	number of breeding places
N	north
E	east
ND4	NADH dehydrogenase subunit 4
COI	cytochrome c oxidase subunit I
cytb	cytochrome b
ITS2	ribosomal internal transcribed spacer 2
dNTP	deoxyribonucleotide triphosphate
ML	maximum likelihood
MP	maximum-parsimony
NJ	neighbour-joining
BIC	bayesian information criterion
GTR	general time-reversible
G	gamma shape parameter
MCMC	markov chain monte carlo
TBR	tree bisection reconnection
BP	bootstrap percentage
K2P	kimura's two-parameter
F1	first generation
bp	basepair
µs/cm	micro Siemens per centimeter
ppt	*parts per thousand*
m	meter
mg/l	milligram per liter
mm	millimeter
rpm	revolutions per minute
nm	nanometer
mM	millimolar
cm	centimeter
ml	milliliter
m^2	square meter
ng	nanogram
pmol	picomoles
U	unit
µl	microliter
s	second
min	minute
h	hour
n	number
ANOVA	analysis of variance
df	degree of freedom
P	possibility value
r	correlation coefficient

α	alpha
β	beta
TMBZ	3,3’5,5’-tetramethylbenzidine
CDNB	1-chloro-2, 4-dinitrobenzene
ACTHI	acetylthiocholine iodide
DTNB	5, 5’-dithiobis -(2-nitrobenzoic acid)
sp.	species (singular)
spp.	species (plural)

CHAPTER 1

GENERAL INTRODUCTION

1.1 SCOPE OF STUDY

In recent decades, infectious diseases carried by mosquito vectors remain a major threat to global public health. To date, about 113 genera and 3,527 species of mosquito have been recorded throughout the world (Jeffery *et al*., 2012). Of the recorded species, there are 442 species of mosquito representing 20 genera documented in Malaysia (Miyagi & Toma, 2000). As far as mosquitoes are concerned, several species of Malaysian mosquito have been incriminated as important public health vectors in diseases transmission, including *Culex quinquefasciatus* Say (Diptera: Culicidae). *Culex quinquefasciatus* is the most abundant and nuisance species (Yap *et al*., 2000a) and its potential as the vector of lymphatic filariasis caused by the nematode parasite *Wuchereria bancrofti* has been acknowledged in Malaysia (Vythilingam *et al*., 2005).

Over the past decades, mosquitoes have been the target of vector control programs due to their significant role in diseases transmission. Mosquito surveillance remains as a preliminary step in mosquito control programs. In Malaysia, a considerable amount of effort has been expended in investigating the distribution of mosquito in larval and adult stages. However, there has been a serious lack of surveillance of *Culex* larvae that primarily live in stagnant water, particularly in East Malaysia. Additionally, little research has been carried out on *Culex* larvae distribution in relation to various habitat characteristics in Southeast Asia region, including Malaysia. Hence, an investigation of the distribution of *Culex* mosquitoes (targeting *Cx. quinquefasciatus*)

and associated habitat characteristics was carried out in the present study by using standardized larval dipping method across 13 states and one federal territory in Malaysia.

Despite the significance of *Cx. quinquefasciatus* in the potential for diseases transmission, little is known about their genetic diversity in Southeast Asia region. In the last few years, a worldwide genotyping of *Cx. quinquefasciatus* has been reported (Fonseca *et al*., 2006). However, Indonesian *Cx. quinquefasciatus* has only been appointed as the sole representative from Southeast Asia and therefore the genetic background of *Cx. quinquefasciatus* from other countries of Southeast Asia should not be disregarded. Molecular phylogeography is an approach for a better understanding of the intraspecific genetic diversity, evolutionary relationship as well as the origins of this mosquito from various localities in Malaysia.

In order to control the widespread of vector-borne diseases, application of organochlorines, organophosphates, carbamates and pyrethroids remains the main method of control in vector control programs. However, extensive usage and over-reliance on insecticides have contributed to the development of insecticide resistance due to the factor of selection pressure (WHO, 2006). Indeed, insecticide resistance is not a new phenomenon and is an increasing problem worldwide. *Culex quinquefasciatus* from different parts of the world have been reported to be resistant to various classes of insecticides (Bisset *et al*., 1997; Chandre *et al*., 1997; Liu *et al*., 2004; Sathantriphop *et al*., 2006; Kasai *et al*., 2007; Pridgeon *et al*., 2008).

WHO larval and adult bioassays are the prerequisite studies for early detection and monitoring of insecticide resistance in the tested population. In addition to identifying the underlying mechanism(s) in insecticide resistance, biochemical microassay is essential for the investigation of the presence of enhanced level in non-specific esterases, mixed function oxidases as well as glutathione-S-transferase

(Hemingway *et al*., 2004). Apart from biochemical analysis, molecular characterization of insecticide resistance has also been illustrated. Around the world, insecticide resistance caused by gene amplification and point mutation has been frequently noted (Paton *et al*., 2000; Hemingway *et al*., 2004). However, molecular characterization of insecticide resistance has not been documented in Malaysia. The present study represents a first attempt to understand the biochemical and molecular basis of insecticide resistance mechanisms in Malaysian mosquitoes.

1.2 SIGNIFICANCE OF STUDY

Vital findings obtained from this study would provide constructive data for medical entomologists, toxicologists, public health stakeholders, epidemiologists, molecular biologists, biochemists as well as other interested parties. The fruit of this study is of paramount importance for the knowledge improvement in vector-borne disease control and management currently practiced in Malaysia. A description of mosquito distribution patterns and breeding preferences according to habitat characteristics provides useful baseline information for local authorities in the justification of the appropriate dosage of larvicide or adulticide in accordance with mosquito species and density. Moreover, the findings of this study could be utilized in the implementation of strategic measures through community participation in environmental manipulation (i.e., to improve drainage systems) as well as elimination of breeding sources.

Besides, the biochemical and molecular characterization of mechanisms underlying the insecticide resistance (i.e., elevated levels of detoxification activities and alteration of target sites) and identification of conserved region of DNA sequences (i.e., mitochondrial, acetylcholinesterase and voltage gated sodium channel) may accelerate the discovery of new molecular targets for the design of more effective insecticides.

1.3 AIMS AND OBJECTIVES

The main goals of this study were to investigate the population structure and insecticide susceptibility status of *Cx. quinquefasciatus* in residential areas across 13 states and one federal territory in Malaysia. With this in mind, the present study was performed to address the following specific objectives:

(1) To determine the distribution of *Cx. quinquefasciatus* and other species of mosquitoes in stagnant water in residential areas in Malaysia.

(i) To probe the infestation rates of *Cx. quinquefasciatus* and other species of mosquitoes in stagnant water in different types of residential areas.

(ii) To investigate the associations between the *Culex* larvae distribution and various habitat characteristics.

(iii) To elucidate the nature of co-occurrence among mosquito larvae.

(2) To investigate the genetic diversity of *Cx. quinquefasciatus*.

(i) To determine the intraspecific genetic diversity, evolutionary relationships and dispersal patterns of Malaysian *Cx. quinquefasciatus*.

(ii) To reveal the phylogeographic relationships between the haplotype and country of origin of *Cx. quinquefasciatus*.

(3) To quantify the insecticide susceptibility status of Malaysian *Cx. quinquefasciatus* populations.

(i) To evaluate the susceptibility status of Malaysian *Cx. quinquefasciatus* against four active ingredients (i.e., DDT, propoxur, malathion and permethrin) representing four major insecticide classes by WHO larval and adult bioassays.

(ii) To correlate the degree of insecticide resistance among four insecticide classes in larval and adult stages.

(4) To characterize the biochemical mechanisms of insecticide resistance in Malaysian *Cx. quinquefasciatus*.

(i) To evaluate the levels of enzyme activities in Malaysian *Cx. quinquefasciatus*.

(ii) To correlate the degree of insecticide resistance with the levels of enzyme activities in Malaysian *Cx. quinquefasciatus*.

(5) To characterize the molecular mechanisms of insecticide resistance in Malaysian *Cx. quinquefasciatus*.

(i) To determine the prevalence of G119S mutation in acetylcholinesterase gene of Malaysian *Cx. quinquefasciatus*.

(ii) To determine the prevalence of L1014F and L1014S mutations in voltage gated sodium channel gene of Malaysian *Cx. quinquefasciatus*.

(iii) To evaluate the associations between the frequency of resistance alleles and the degree of insecticide resistance in Malaysian *Cx. quinquefasciatus*.

A schematic flowchart of the study is illustrated in Figure 1.1.

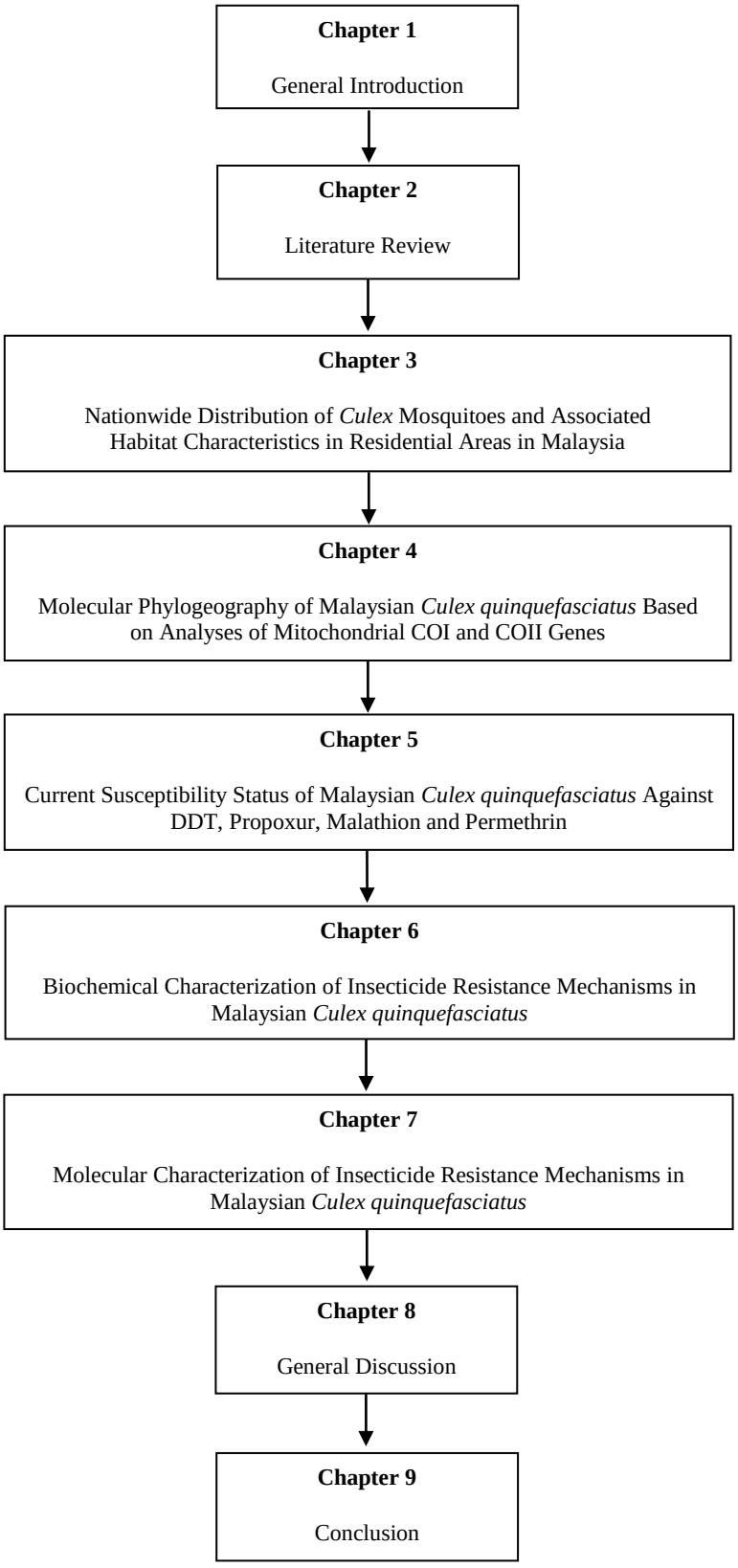

Figure 1.1 Schematic diagram of "Nationwide Distribution and Insecticide resistance Study of Malaysian Mosquito *Culex quinquefasciatus* Say by Molecular and Biochemical Tools"

CHAPTER 2

LITERATURE REVIEW

2.1 BIOLOGY OF *CULEX QUINQUEFASCIATUS* SAY

Mosquitoes are members of the Family Culicidae in the Order Diptera. Around the world, over 3,500 species and subspecies of mosquitoes have been recorded. The majority of mosquito species fall into three groups, namely anophelines, culicines and aedines (Eldridge, 2005). The classification for *Cx. quinquefasciatus* Say is described below:

Phylum : Arthropoda

Class : Insecta

Order : Diptera

Family : Culicidae

Subfamily : Culicinae

Tribus : Culicini

Genus : *Culex*

Subgenus : *Culex*

Species : *Culex quinquefasciatus*

(Knight & Stone, 1977)

Culex quinquefasciatus was described by Thomas Say (1823). *Culex fatigans* is the junior synonym of *Cx. quinquefasciatus*. However, the name *quinquefasciatus* Say (1823) was recognized to have precedence over *fatigans* Wiedemann (1928) (Harbach, 2012). *Culex quinquefasciatus* is one of the members of *Cx. pipiens* complex. This group of species complex consists of several sibling species, including *Cx. pipiens* Linnaeus, *Cx. quinquefasciatus* Say, *Cx. pipiens pallens* Coquillett, *Cx. pipiens australicus* Dobrotworsky and Drummond and *Cx. pipiens* form *molestus* Forskal (Fonseca *et al*., 2004; Smith & Fonseca, 2004; Kent *et al*., 2007; Harbach, 2012).

Literature suggested that *Cx. quinquefasciatus* is native to Africa (Vinogradova, 2000). Through human activities, it has been broadly spread throughout tropical, subtropical and warm temperate regions (Vatandoost *et al*., 2004). Its wide range of distribution has also been documented in Thailand (Kitvatanachai *et al*., 2005), Florida (Kline *et al*., 2006), Georgia (Calhoun *et al*., 2007), Argentina (Gleiser & Zalazar, 2010) and India (Kaliwal *et al*., 2010). *Culex quinquefasciatus* is a night time biter that feeds on human and animals. It is the most common domestic species in urban, sub-urban and rural areas, where 53.2 - 62.7% were anthropophilic (Reuben, 1992). They are inactive at daytime and used to rest in dark corner of rooms, shelters, culverts as well as in the vegetations and tree holes (Rozandaal, 1997).

In general, *Culex* mosquitoes (including *Cx. quinquefasciatus*) take around 14 days to complete their life cycle. The female lays rafts of 100 or more eggs in pools of water after two to three days of blood-feeding. In tropical regions, the eggs hatch within two to three days while in temperate regions, they may not hatch until after one or two weeks. *Culex* mosquitoes breed on a large variety of water surface such as artificial containers, stagnant drainage water, underground systems, septic tanks, pit latrines, blocked canals and abandoned wells. The emerging larvae feed on algae, bacteria and other organic matters in the water. The larval stage lasts seven days followed by the

pupal stage that lasts two to three days and finally emerges to adult stage. The adult stage lasts one to two weeks but also hibernates in protected locations during the winter (Rozandaal, 1997; Pimentel, 2004; Service, 2004).

Culex mosquitoes could be easily differentiated from other genera of mosquitoes at egg, larval as well as adult stages. *Culex* mosquitoes lay a raft of eggs on water surface while other genera of mosquitoes lay their eggs individually. In larval stage, *Culex* larvae use siphon for breathing. *Aedes* larvae have shorter siphons compared to *Culex* larvae while *Anopheles* larvae do not have siphons. In adult stage, the length of the maxillary palps is less than half of the length of the proboscis and they rest parallel to the surface (Yap *et al*., 2000a).

Culex quinquefasciatus larvae have a patch of 30-40 comb scales, 10-12 teeth on each side of the mental plate and a siphon with length about one third of its base. Meanwhile, *Cx. quinquefasciatus* adults have a pale scaled and a few dark scaled patches extending towards the middle of abdominal sternites (Belkin, 1968).

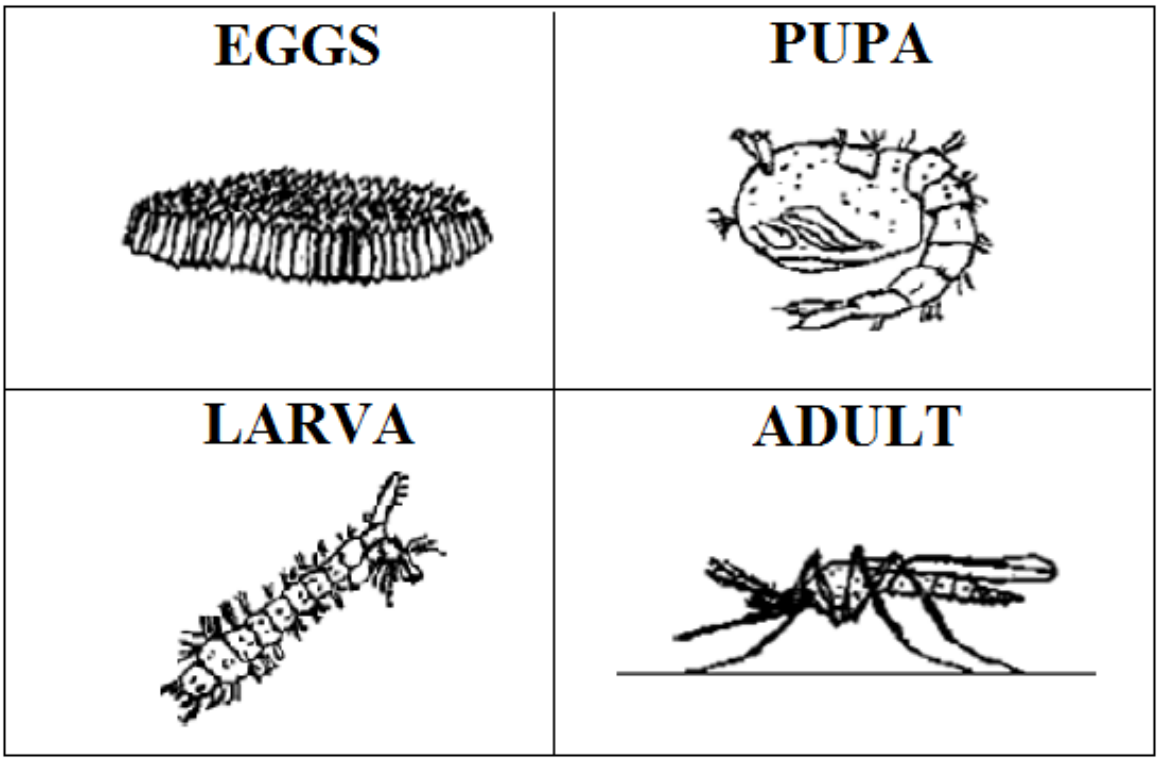

Figure 2.1 *Culex* mosquito eggs, larva, pupa and adult.

(Pimentel, 2004)

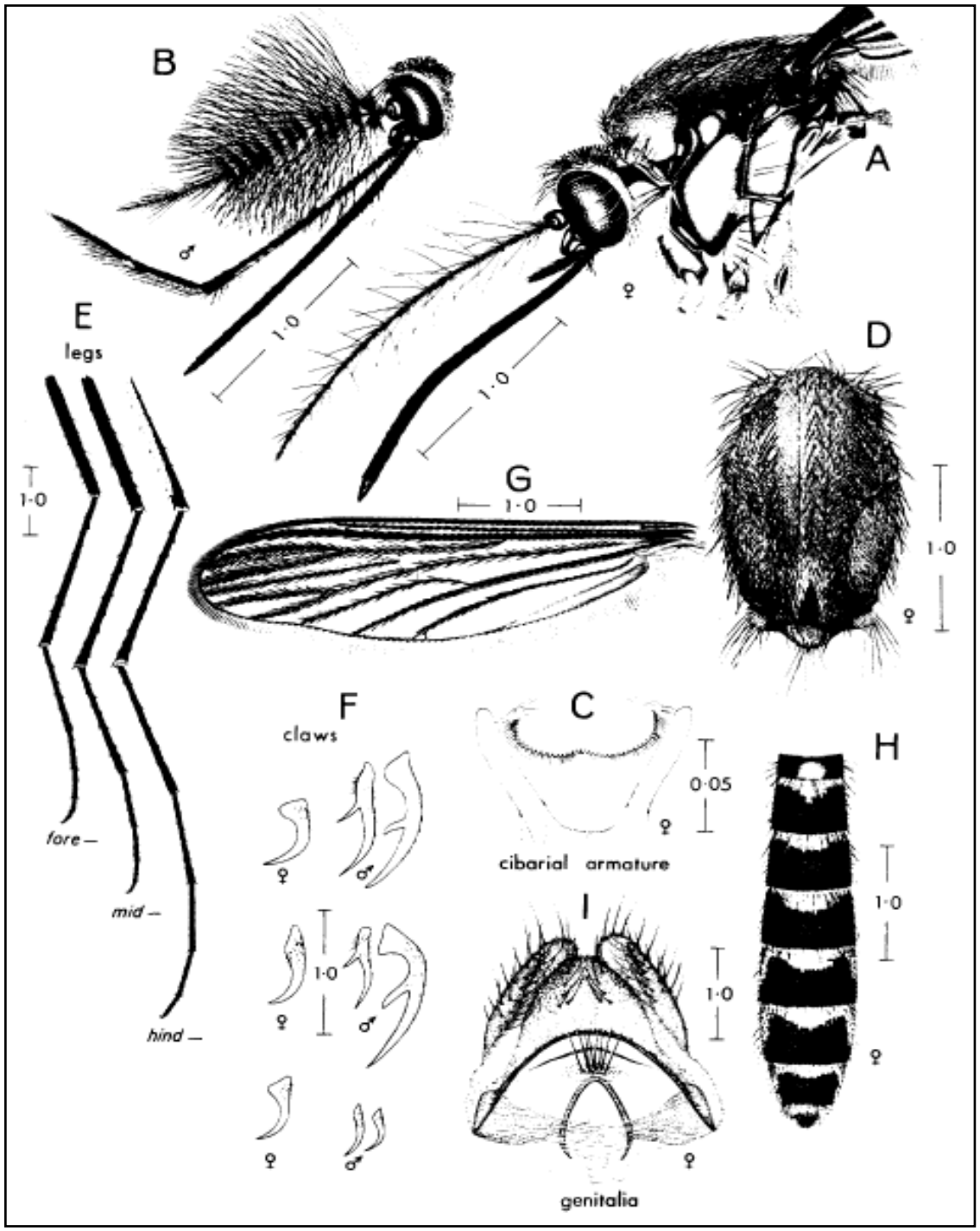

Figure 2.2 *Culex quinquefasciatus* adult (A) female head and thorax, lateral view; (B) male head, lateral view; (C) female cibarial armature; (D) female thorax, dorsal view; (E) legs, anterodorsal views; (F) male, female tarsal claws; (G) wing, dorsal view; (H) female abdomen, dorsal view; (I) female genitalia.

(Sirivanakar & White, 1978)

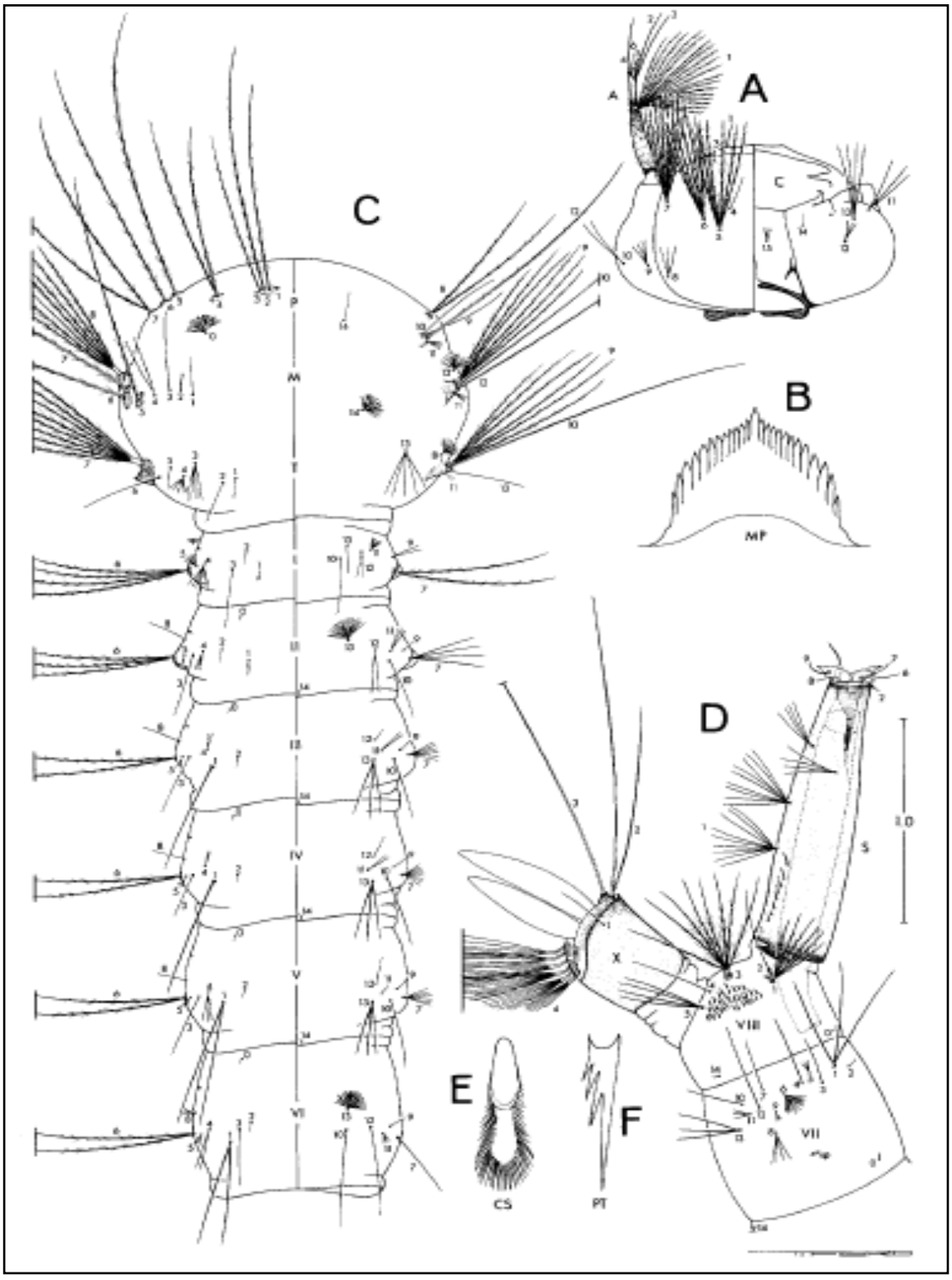

Figure 2.3 *Culex quinquefasciatus* larva. (A) head; (B) mental plate; (C) thorax and abdomen I-VI; (D) abdomen VII, VIII, siphon and saddle; (E) comb scale; (F) pecten tooth.

(Sirivanakar & White, 1978)

2.2 MEDICAL AND VETERINARY IMPORTANCE OF *CULEX QUINQUEFASCIATUS* SAY

WHO (1996a) has declared mosquito as "public enemy number one". There are over 3,500 species of mosquitoes, of which more than 100 species of mosquitoes are vectors of human diseases (Rozandaal, 1997). In Malaysia, *Cx. quinquefasciatus* is a potential vector of bancroftian filariasis (Vythilingam *et al*., 2005). Around the world, its significance in vector-borne diseases has been well-documented. *Culex quinquefasciatus* has been reported as an important vector of Japanese encephalitis in Asia such as Thailand (Nitatpattana *et al*., 2005), Vietnam (Do *et al*., 1994) and India (Mourya *et al*., 1989). Besides, it is the most important vector of filariasis in India (Das, 1976; Mahanta *et al*., 2001; Samuel *et al*., 2004). It is also a primary vector of Saint Louis encephalitis (Jones *et al*., 2002; Flores *et al*., 2010) and West Nile virus in United States (Parker *et al*., 2011). In Australia, it has been incriminated as the vector of Ross River virus (Lindsay *et al*., 1993).

In light of veterinary importance, it is an important vector of avian malaria protozoa, *Plasmodium relictum* in Hawaii (Fonseca *et al*., 2000; LaPointe *et al*., 2005). Moreover, its significance in the transmission of *Dirofilaria immitis* (dog heartworm) has also been acknowledged in Taiwan (Wu *et al*., 1997; Lai *et al*., 2001) and Brazil (Ahid *et al*., 2000). It is also a potential vector of myxomatosis and reticuloendotheliosis in New Zealand (Holder, 1999).

2.3 POPULATION STUDIES OF MOSQUITO VECTORS IN MALAYSIA

Mosquito population studies remain as a preliminary step for the monitoring and control of vector. The population studies of mosquito larvae and adults using different approaches have been frequently reported in Malaysia. The density and abundance of mosquito vectors have been carried out in Malaysia by using Human Landing Catches (Reid, 1961; Parsons *et al*., 1974; Hii & Vun, 1985; Rohani *et al*., 2008; Tan *et al*., 2008; Ali *et al*., 2011). Distribution of mosquito studies using animal-baited traps have also been reported (Reid, 1961; Rohani *et al*., 1999; Tan *et al*., 2008). In addition to identifying the mosquito breeding sites, container surveys have been conducted (Hassan *et al*., 2005; Cheah *et al*., 2006; Chen *et al*., 2009; Nyamah *et al*., 2010). The studies of mosquito density by using light traps have also been documented (Parsons *et al*., 1974; Vythilingam *et al*., 1992; Oli *et al*., 2005). Among numerous studies focusing on dengue vector which were published in recent years, ovitrap surveillance is the most widely used method (Chen *et al*., 2005a; 2006a; Cheah *et al*., 2006; Rozilawati *et al*., 2007; Wan-Norafikah *et al*., 2009; Lim *et al*., 2010; Norzahira *et al*., 2011; Rohani *et al*., 2011). Larval dipping is another approach for the population study as well as the investigation of mosquito breeding site. However, the study of larval dipping surveillance in Malaysia is limited, particularly in East Malaysia. In 2010, Rohani *et al*. reported the abundance of mosquito larvae in the district of Pahang, while Hassan *et al*. (2005, 2010) reported the abundance of *Culex* larvae in the district of Penang, by using dipping method. With regard to the population genetic structure of Malaysian mosquitoes, considerable efforts have been made in studies of several species of *Anopheles* mosquitoes (Walton *et al*., 2007; Morgan *et al*., 2011) and *Ae. albopictus* (Birungi & Munstermann, 2002), whereas mosquitoes in other genera have not yet been studied.

2.4 MOLECULAR PHYLOGEOGRAPHY OF MOSQUITO VECTORS BASED ON MITOCHONDRIAL DNA

Phylogeography is defined as "the field of study concerned with the principles and processes governing the geographical distribution of genealogical lineages, especially those at the intraspecific level" (Beebee & Rowe, 2008). Mitochondrial DNA is the most widely used marker for the study of molecular ecology in animal taxa (Simon *et al*., 1994) and it is recognized as the standard phylogeographic tool (Beebee & Rowe, 2008). Animal mitochondrial DNA has been proven to be an ideal molecular marker due to its uniparental inheritance, lack of recombination and higher rate of mutation (Lowe *et al*., 2004). The molecule of mitochondrial DNA consists of 37 genes coding for 2 rRNA, 13 mRNA and 22 tRNA with major coding regions such as ND1, ND2, ND4, ND5, ND6, COI, COII, COIII, Cyt b, ATP, N3, 16S and 12S (Clary & Wolstenholme, 1985).

Among the mitochondrial DNA, ND4, ND5, COI, COII and 16S genes have been by far the most popular markers in the studies of molecular phylogeography. Based on ND4 gene, three haplotypes from Peruvian *Ae. aegypti* populations (da Costa-da-Silva *et al*., 2005), eight haplotypes from African *An. nili* populations (Ndo *et al*., 2010) and 20 haplotypes from Brazilian *An. darlingi* populations (Angêla *et al*., 2007) were identified. As for ND5 gene, a reduced level of genetic diversity in *Ae. albopictus* populations in Brazil have been reported by Maia *et al*. (2009), where only two haplotypes were detected. However, in comparison with the populations from the United States and Asia, a total of nine haplotypes were detected. Moreover, ND5 gene also revealed a total of 13 haplotypes in *Ae. albopictus* populations from Brazil, Malaysia, Madagascar, Indonesia, Japan, Hawaii and Cameroon (Usmani-Brown *et al*., 2009). Based on COI gene, four haplotypes in *Ae. albopictus* populations in Cameroon

(Kamgang *et al*., 2011) and East-Adriatic (Zitko *et al*., 2011) and 12 haplotypes in *An. darlingi* populations in Colombia (Gutiérrez *et al*., 2010) were identified. In regard to COII gene, two haplotypes in *Cx. quinquefasciatus* from Bangladesh (Hasan *et al*., 2009), seven haplotypes in *An. nili* from Africa and 26 haplotypes in *An. farauti senso stricto* from Melanesia (Hasan *et al*., 2008) were found. In addition, 16S rRNA gene revealed that Indian *Cx. quinquefasciatus* populations were genetically diverse (Sharma *et al*., 2010).

Based on these intraspecific markers, both COI and COII genes have been reputed as reliable and useful genetic markers in the study of Walton *et al*. (2000) and Chen *et al*. (2004), by revealing 70 haplotypes of *An. dirus* from 14 study sites (84 individuals) throughout Thailand, Myanmar and Bangladesh; 50 haplotypes of *An. jeyporiensis* from 16 study sites (76 individuals) throughout southern China and northern Vietnam, respectively.

2.5 INSECTICIDES

Generally, insecticides fall into four major classes, namely organochlorine, carbamate, organophosphate and pyrethroid. Newer insecticides such as organophosphate, carbamate and pyrethroid have been introduced as alternatives to chlorinated hydrocarbon insecticide. To date, pyrethroid is the most important class of insecticide with major usage in public health and household insecticide products (Yap *et al*., 2000b).

Adulticides are specifically designed in ultra low volume (ULV) fogging, thermal fogging, surface residual spray and household insecticide products for the control of adult mosquitoes (Yap *et al*., 2000b). On the other hand, larvicides are the insecticide that is designed for control of immature stages of mosquitoes, especially the

larva stage (WHO, 1996b). More recently, insect growth regulators (juvenile hormone mimics and chitin synthesis inhibitors) have also been introduced but not widely used due to the slow action and lower effectiveness of these larvicides for the control of younger mosquito larvae (Yap *et al*., 2000b). Hence, application of the conventional insecticides remains as the most common strategy in vector control programs.

2.5.1 DDT

DDT (1,1,1 -trichloro-2,2-bis(chlorophenyl)ethane) belongs to the class of organochlorine. The structural formula of DDT is shown in Figure 2.4. It was first synthesized in 1874 and its insecticidal properties have been discovered in 1939 (WHO, 1979). DDT is effective, relatively inexpensive to manufacture and persist in the environment (Klaassen *et al*., 1996). DDT attacks the nervous system of insects by interfering with normal nerve impulses (Klaassen *et al*., 1996). In 1972, DDT was banned due to its carcinogenicity, bioaccumulation and health effects on non-target organisms (EPA, 1990; NPIC, 1999). However, the exception for the usage of DDT is only restricted for the control of vector-borne diseases, particularly for malaria vector control (NPIC, 1999; Zaim, 2002). In Malaysia, DDT application in vector control programs was stopped in 1997 (Zaim, 2002).

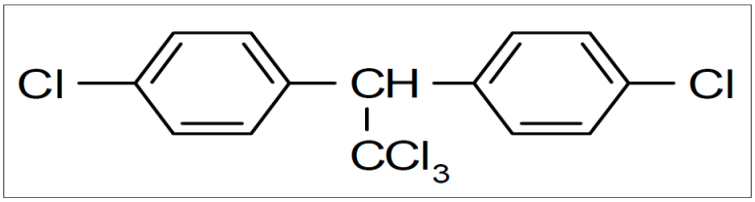

Figure 2.4 Structural formula of DDT.

(WHO, 2009a)

2.5.2 PROPOXUR

Propoxur (2- isopropoxyphenyl methylcarbamate) belongs to the class of carbamate and it was first introduced in 1959 (Hayes & Laws, 1990). The structural formula of propoxur is shown in Figure 2.5. Propoxur is commonly used for the control of agricultural and public health pests. It interferes with nervous transmission across the synaptic gap through inhibition of acetylcholinesterase. Unlike DDT, propoxur does not accumulate in tissues and to date there is no evidence that propoxur is carcinogenic (WHO, 2005). The worldwide usage of carbamates (i.e., propoxur and bendiocarb) has been limited and about 50% of the usage were in Southeast Asian countries (Zaim, 2002).

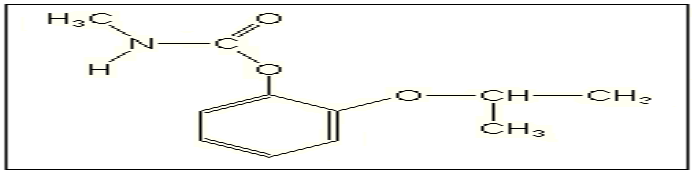

Figure 2.5 Structural formula of propoxur.

(WHO, 2005)

2.5.3 MALATHION

Malathion (S- 1,2- bis(ethoxycarbonyl)ethyl O,O- dimethyl phosphorodithioate) is an organophosphate insecticide and it was first introduced in 1950 (Bonner *et al*., 2007). The structural formula of malathion is shown in Figure 2.6. It is a broad-spectrum insecticide that could be utilized to control a wide range of outdoor insect pests (NPIC, 2010). Malathion binds with enzyme acetylcholinesterase at nerve endings of the insects which causes overstimulation of nerve signals and consequently leads to paralysis and

death (Reigart & Roberts, 1999; NPIC, 2010). Before 1995, organophosphates (mainly malathion) have been widely applied in dengue vector control in Malaysia (Zaim, 2002).

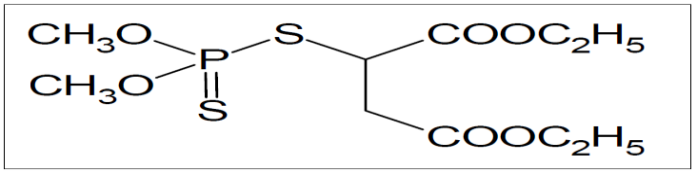

Figure 2.6 Structural formula of malathion.

(WHO, 2003)

2.5.4 PERMETHRIN

Permethrin (3- phenoxybenzyl(1RS,3RS;1RS,3SR)- 3- (2,2- dichlorovinyl)- 2,2 dimethyl-cyclopropanecarboxylate) is a pyrethroid insecticide. Permethrin was first synthesized in 1973 and introduced into markets in 1977 as a photostable pyrethroid (Elliott *et al*., 1973). The structural formula of permethrin is shown in Figure 2.7. Permethrin has been widely used in mosquito control programs and commonly applied in ornamental lawns, livestock, pets, residential areas as well as restaurants (NPIC, 2009). Permethrin attacks the nervous system of insects by interfering with sodium channel to disrupt the functioning of neurons which causes muscles to spasm and eventually death. In Malaysia, permethrin has been frequently used since 1996 for the control of malaria and dengue vectors (Nazni *et al*., 1998; Zaim, 2002).

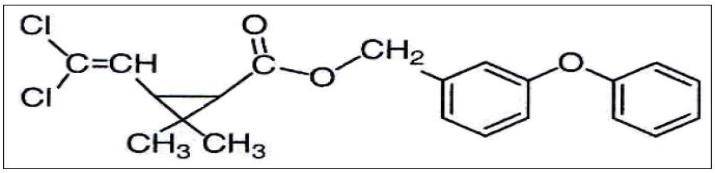

Figure 2.7 Structural formula of permethrin.

(WHO, 2009b)

2.6 INSECTICIDE RESISTANCE

Application of insecticides is a common strategy for insect pest control. Nevertheless, over-reliance of insecticide often causes resistant strain to evolve in the field populations. In the mid-nineteenth century, insecticide resistance is defined as "the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species" (WHO, 1957). However, this definition reflects a population view rather than a focus on single individuals (Whalon *et al*., 2008). Due to the genetics of selection, the definition has been refined as 'resistance marks a genetic change in response to selection by toxicants that may impair control in the field' (Sawicki, 1987), which also reflects the survival of single individuals within populations.

The degree of insecticide resistance in insect populations is associated with the volume and frequency of insecticide application, in conjunction with the inherent characteristics (i.e., life cycle) of the particular insect species (Hemingway & Ranson, 2000). To date, a total of 7,747 cases of resistance with more than 331 insecticide compounds have been reported in 553 species of arthropod (Whalon *et al*., 2008) (Figure 2.4). Specifically, more than 100 mosquito species have developed insecticide resistance against various classes of insecticides (Hemingway & Ranson, 2000). In point of fact, the insecticide-resistant vectors of public health importance and the occurrence of multiple or cross insecticide resistance are both increasing problems in many parts of the world and have become a major obstacle in vector control programs (WHO, 2006). It has been proposed that the shorter life cycles with abundant progeny in mosquitoes give rise to rapid insecticide resistance evolution (Hemingway & Ranson, 2000). Apart from that, many researchers have found that insecticide resistance cases

found in the mosquito populations were due to the factor of selection pressure (Liu *et al*., 2004; WHO, 2006).

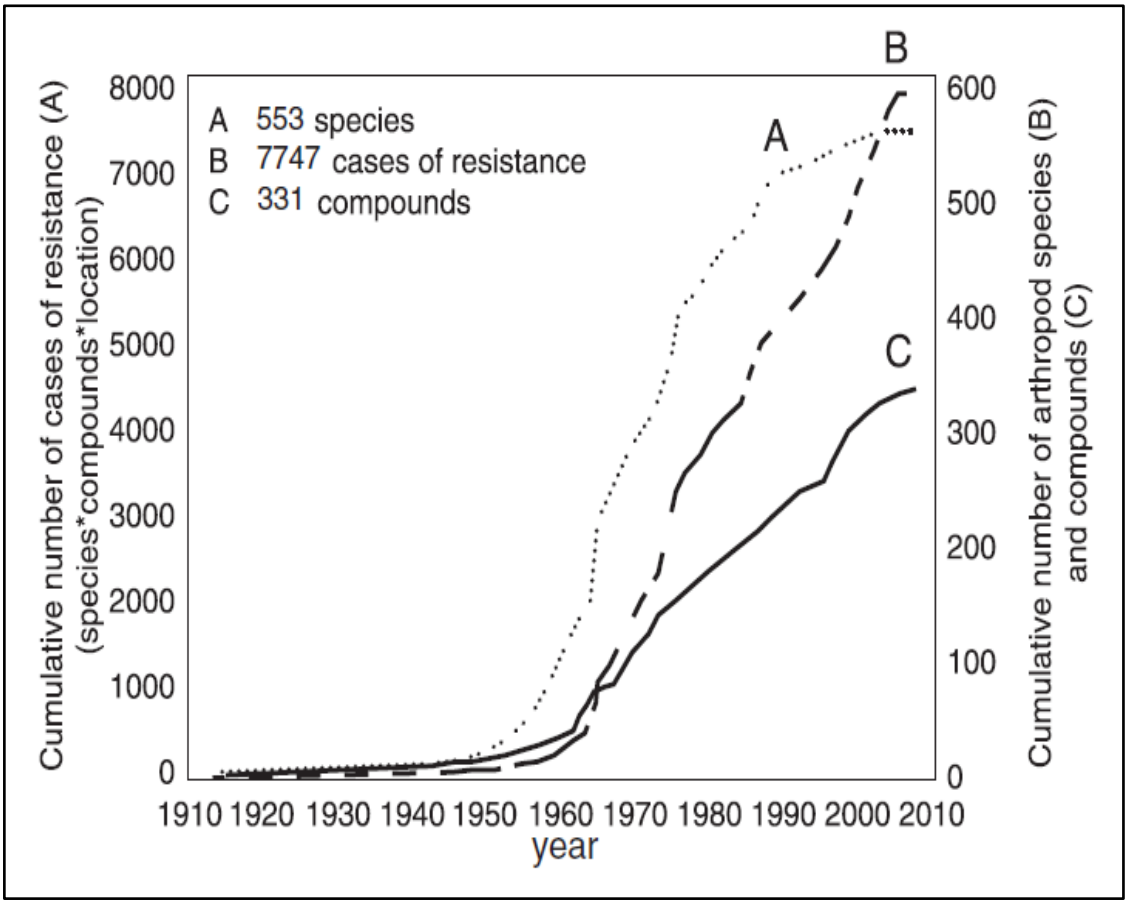

Figure 2.8 A century of evolution of arthropod insecticide resistance.

(Whalon *et al*., 2008)

2.7 INSECTICIDE RESISTANCE MECHANISMS

Generally, insecticide resistance mechanisms could be classified into several groups: increased metabolic detoxification and alteration of target site, decreased rates of penetration and behavioral changes in insects (Hemingway *et al*., 2004; Hollingworth & Dong, 2008, Karaagac, 2012).

2.7.1 INCREASED METABOLIC DETOXIFICATION

2.7.1.1 ESTERASES (EST)

Most of the insecticides used are esters (i.e., organophosphates, carbamates and pyrethroids). The hydrolysis of ester group causes a significant decrease in toxicity. As a consequence, EST activity plays an important role in insecticide resistance (Hollingworth & Dong, 2008). Biochemical analyses revealed that elevated non-specific EST confer organophosphate resistance (Brogdon & Barber, 1990; Bisset *et al*., 1990; Peiris & Hemingway, 1990; Pethuan *et al*., 2007; Swain *et al*., 2009), carbamate resistance (Peiris & Hemingway, 1993) and pyrethroid resistance (Vulule *et al*., 1999; Matowo *et al*., 2010; Brogdon & Barber, 1990).

On the other hand, an elevated level of EST through gene amplification or upregulation has also been involved in resistance to organophosphates, carbamates and pyrethroids (Bass & Field, 2011). EST gene amplification such as $est\alpha2^1$, $est\beta2^1$ (DeSilva *et al*., 1997; Paton *et al*., 2000) as well as $est\alpha^3$ (DeSilva *et al*., 1997) have been identified. It has been reported that co-amplification of both $est\alpha2^1$ and $est\beta2^1$ were commonly found in organophosphate-resistant *Cx. quinquefasciatus* (Hemingway *et al*., 2004).

2.7.1.2 MIXED FUNCTION OXIDASES (MFO)

MFOs are a complex family of enzymes and it has been evidenced that different enzymes produce resistance to different classes of insecticides (Karunaratne, 1998). An increased level of mixed function oxidases could contribute resistance to organochlorines, carbamates, organophosphates and pyrethroids (Brewer & Keil, 1989; Brooke *et al*., 2001; Fonseca-González *et al*., 2009). Specifically, the multifunctional monooxygenases catalyzed by cytochrome P450, a member of the MFO system, comprise the major metabolisms of insecticides and play a major role in insecticide resistance (Hollingworth & Dong, 2008). Molecular characterization has evidenced that a number of these cytochrome P450 genes are associated with insecticide resistance, such as *CYP6D1*, *CYP6A1*, *CYP6A2*, *CYP6B2*, *CYP4G8* and *CYP6A8* (Karunaratne, 1998; Hemingway & Ranson, 2000; Hemingway *et al*., 2004).

2.7.1.3 GLUTATHIONE-S-TRANSFERASES (GST)

GSTs are a diverse family of enzymes that strengthen the reaction of the cycteine sulphydryl group of the tripeptide glutathione (GSH) with xenobiotics, including insecticides (Hollingworth & Dong, 2008). The involvement of GST in pyrethroid resistance (Che-Mendoza *et al*., 2009), organophosphate resistance (Hemingway *et al*., 1991) and organochlorine resistance (Prapanthadara *et al*., 1993, 1996; Hemingway, 2000, Lumjuan *et al*., 2005; Zayed *et al*., 2006) has been frequently reported. The *Gst2* and *Gstt-6a* genes that conferred insecticide resistance have also been identified (Hollingworth & Dong, 2008).

2.7.2 ALTERATION OF TARGET SITE

2.7.2.1 INSENSITIVITY OF SODIUM CHANNEL

Both pyrethroids and DDT are specifically designed to attack the voltage gated sodium channel which consists of four domains (I-IV) while each domain comprising six transmembrane helices (S1-S6) (Hemingway *et al*., 2004). Altered sodium channel causes DDT and pyrethroid resistance (also known as knockdown resistance, *kdr*) in a wide range of insect species and this knockdown resistance was first identified in house fly, *Musca domestica* in 1950's (Soderlund & Knipple, 2003). The *kdr* mutation at position 1014, leusine to phenylalanine substitution (L1014F) (Martinez-Torres *et al*., 1998, 1999; Matambo *et al*., 2007; Liu *et al*., 2009; Chen *et al*., 2010) and leusine to serine substitution (L1014S) (Martinez-Torres *et al*., 1999; Chen *et al*., 2010) in the sodium channel gene have been frequently reported. It has been documented that mosquitoes with 1014F mutation contributed high levels of resistance against both DDT and pyrethroids, while the 1014S mutation contributed high levels of resistance against DDT but low levels of resistance against pyrethroids (Martinez-Torres *et al*., 1999). In addition, a combination of L1014F substitution and M918T substitution, called *super-kdr* gene has been reported in housefly (i.e., *Musca domestica*) and horn fly (i.e., *Hematobia irritans*) (Soderlund & Knipple, 2003; Hemingway *et al*., 2004). To date, at least 20 sodium channel amino acid sequence polymorphisms that involved in knockdown resistance have been identified and all mutations identified in mosquitoes have only been found in domain II of the sodium channel (Soderlund & Knipple, 2003).

2.7.2.2 INSENSITIVITY OF ACETYLCHOLINESTERASE

Both organophosphates and carbamates act on the acetylcholinesterase of insects (Hollingworth & Dong, 2008). Early studies have employed biochemical method to characterize the alteration of acetylcholinesterase and determine the frequencies of resistance genotype of insects in a heterogeneous population (Hemingway *et al*., 1986; WHO, 1998). Recent advances in molecular biology have unraveled several insensitive acetylcholinesterase genes. The *ace-1* gene, amino acid substitution glycine to serine at position 119 (G119S) that caused insecticide resistance has been well-documented (Cui *et al*., 2006; Alout *et al*., 2007, 2011; Djogbénou *et al*., 2008). Besides, an amino acid substitution, phenylalanine to tryptophan at position 455 (F455W) in *ace-2* gene has been reported as well (Nabeshima *et al*., 2004). Moreover, mutations V180L, G262A, G262V, F237Y and G365A which caused different degree of insecticide resistance have also been discovered (Walsh *et al*., 2001). Previous studies evidenced that an altered acetylcholinesterase in mosquitoes lead to high carbamate and low organophosphate resistance (Hemingway *et al*., 2004).

2.7.2.3 INSENSITIVITY OF GAMMA AMINOBUTYRIC ACID RECEPTOR

Gamma aminobutyric acid (GABA) receptor is the target site of cyclodiene insecticides (i.e., dieldrin). It has been documented that an amino substitution, alanine to serine at position 302 confers resistance to dieldrin (known as *Rdl* gene) (Hemingway *et al*., 2004) and this mutation was first identified in *Drosophila* (Hemingway & Ranson, 2000). The replacement of alanine to serine or glycine at position 302 has been reported in a number of insect species (Hemingway & Ranson, 2000; Hemingway *et al*., 2004). As far as mosquitoes are concerned, the *Rdl* gene has been first identified in *Ae. aegypti*

in 1993 (Thompson *et al*., 1993). Subsequently, the identification of other mutations in *Rdl* gene (i.e., A302S and A302G) has only been recently described in other species of mosquitoes such as *Ae. albopictus, Cx. quinquefasciatus* (Tantely *et al*., 2010) and *Anopheles* mosquitoes (Asih *et al*., 2012).

2.7.3 OTHER MECHANISMS OF INSECTICIDE RESISTANCE

The reduced rate of insecticide penetration is one of the insecticide resistance mechanisms where the resistant insects absorb the insecticide more slowly than susceptible insects as the insect cuticle has developed a barrier which causes the slow absorption of the chemicals into body (Karaagac, 2012). Over the years, penetration resistance has been documented in house flies (Wen & Scott, 1999), cockroaches (Valles *et al*., 2000), moths (Ahmad *et al*., 2006) and beetles (Zhang *et al*., 2008) as well as mosquitoes (Wood *et al*., 2010). In addition, behavioral resistance has also been described as one of the insecticide resistance mechanisms. Behavioral resistance is defined as 'the developments of behaviors that reduce an insect's exposure to a toxin or that allow an insect to survive in an environment that is harmful and/or fatal to the majority of other insects' (Sparks *et al*., 1989). To reduce the rate of insecticide contact in indoor environment, changes in house entry/exit rate and feeding time of mosquitoes have been observed (Mbogo *et al*., 1996; Mathenge *et al*., 2001).

2.8 DIAGNOSING INSECTICIDE-RESISTANT MOSQUITOES

The assessment of insecticide resistance status could be classified into two categories: (1) *in vivo* technique (i.e., dose-response and diagnostic dosage) on intact individuals which involves the exposure of insecticide and (2) *in vitro* technique (i.e., enzyme

microplate assay, protein electrophoresis and polymerase chain reaction detection) which investigates the enzyme activities or the quantity of DNA coding for specific resistance genes in insects. Both *in vivo* and *in vitro* techniques often regarded as complementary and mutually dependent (ffrench-Constant & Roush, 1991).

2.8.1 WHO SUSCEPTIBILITY TESTS

WHO larval and adult susceptibility bioassays (1981a, 1981b) are the standard measures for early detection of insecticide resistance in mosquito populations. Adult and larval bioassays results are subjected to probit analysis. Based on the value of LC_{50} (50% lethal concentration, known for larval bioassay) and KT_{50} (50% knockdown time, known for adult bioassay), resistance ratio (RR) is determined by the ratio of resistant strain to the ratio of susceptible strain, by adopting the method of Brown & Pal (1971). Calculated RR values 10 are indicative of high resistance, 5-10 are indicative of medium resistance and > 5 are indicative of low resistance (Mazarri & Georghiou 1995). With regard to adult bioassay, the percentage mortality at 24 hours post-treatment, which in 98-100% mortality indicates susceptibility, 80-97% mortality suggests the possibility of resistance that needs to be further confirmed and less than 80% mortality suggests resistance (WHO, 2009c).

The determination of LC_{50} in larval bioassay is able to detect the resistance at high frequencies, but not efficient in detecting the small changes in resistance frequency, especially when the resistance is first emerging in the population (Roush & Miller, 1986; ffrench-Constant & Roush, 1991). On the other hand, the diagnostic dosages in adult bioassay are more sensitive in detecting low resistance frequencies as an appropriate concentration is applied to all tested individuals (ffrench-Constant & Roush, 1991).

Both larval and adult bioassays are labor intensive and the frequency of resistance gene could not be estimated in mosquito populations (Zayed *et al*., 2006).

2.8.2 ENZYME MICROASSAYS

The involvement of acetylcholinesterase, esterase, glutathione-S-transferase and oxidases in insecticide resistance has been frequently reported (Hemingway & Karunaratne, 1998; Hemingway *et al*., 2004; Zayed *et al*., 2006). Enzyme microassay has been commonly used due to its rapid, simple and sensitive method for the identification of mechanisms underlying the insecticide resistance in mosquito populations even at low frequencies (Brogdon, 1989; Lee, 1990). In addition, enzyme microassay could confirm the results of bioassay and identify the specific resistance mechanism (Zayed *et al*., 2006). Multiple assays could be performed on a single insect for the detection of multiple resistance (Brown & Bradgon, 1987). Moreover, the actual mechanism(s) that conferred the occurrence of cross-resistance could be identified (Brooke *et al*., 2001; Cuamba *et al*., 2010). Microassay could be used to determine the resistance gene frequencies under different selection pressure (WHO, 1998).

Based on the mean enzyme level, resistance ratios (RR) were calculated by dividing values of the field strain by those of the laboratory reference strain. Calculated RR values > 1 are indicative of resistance, while values ≤ 1 are indicative of susceptible (Chen *et al*., 2008).

2.8.3 PROTEOMICS ANALYSES

Native polyacrylamide gel electrophoresis (PAGE) has been commonly used for the identification of specific esterases (WHO, 1998). Based on the qualitative analysis, the electrophoretic studies could demonstrate the enzyme esterase band patterns of mosquitoes (Selvi *et al*., 2010). The level of insecticide resistance is associated with the number of esterase band (Chen & Sudderudin, 1987). Intensity of stain color could be used as the indication of enzyme activity levels that conferred insecticide resistance. The darker color of band indicated higher enzyme activity (Grafton-Cardwell *et al*., 1998). In recent years, 2D-electrophoresis has been adopted for the characterization of insecticide resistance mechanisms in agricultural pests such as fruit flies (Pedra *et al*., 2005; Alias & Clark, 2010; Jin *et al*., 2012) and whiteflies (Kang *et al*., 2012) while this technique has not been commonly applied in mosquitoes.

2.8.4 POLYMERASE CHAIN REACTION (PCR) DETECTION

Insecticide resistance mechanisms could be detected by PCR such as mutations in sodium channel, acetylcholinesterase and GABA receptors (Hemingway *et al*., 2004) as well as gene amplification and duplication (i.e., esterases, glutathione-S-transferases and cytochrome P450 monooxygenases) (Hemingway & Karunaratne, 1998; Paton *et al*., 2000; Bass & Field, 2011). The gold standard method, DNA sequencing could be used to detect the presence of resistance gene, but it is not applicable for large scale population (Hemingway *et al*., 2004). PCR-based diagnostic assays have been developed for a rapid detection of resistance gene in the genome of a single mosquito (Martinez-Torres *et al*., 1998). In recent years, a number of rapid diagnostic resistance markers have been successfully established. PCR-restriction fragment length

polymorphism (PCR-RFLP) (Weill *et al*., 2004; Cui *et al*., 2006) has been employed for the detection of insensitive acetylcholinesterase genotype. As for voltage gated sodium channel genotype, allele-specific-PCR (AS-PCR) (Martinez-Torres *et al*., 1999; Wang *et al*., 2012), hot ligation oligonucleotide assay (HOLO) (Lynd *et al*., 2005), sequence-specific oligonucleotide probes (SSOP) (Kulkarni *et al*., 2006) and fluorescence resonance energy transfer/melt curve analysis (FRET/MCA) (Verhaeghen *et al*., 2006**)** have also been developed. In addition, a multiplex PCR-based assay has been developed for the detection of knockdown resistance gene and insensitive acetylcholinesterase gene simultaneously (Kazanidou *et al*., 2009).

CHAPTER 3

NATIONWIDE DISTRIBUTION OF *CULEX* MOSQUITOES AND ASSOCIATED HABITAT CHARACTERISTICS IN RESIDENTIAL AREAS IN MALAYSIA

3.1 INTRODUCTION

The infectious diseases carried by mosquito vectors have been an increasing public health concern in recent decades. The mosquito-borne diseases and their vectors have been well-documented in every part of the world including Malaysia. There are 442 species of mosquito representing 20 genera recorded in Malaysia (Miyagi & Toma 2000). Several species of Malaysian mosquitoes have been incriminated as important public health vectors in disease transmission. In this region, *Ae. aegypti* and *Ae. albopictus* are the dengue vectors (Lee & Inder 1993); *Cx. gelidius* is the principal vector of Japanese encephalitis (Vythilingam *et al*., 1994). *Mansonia uniformis*, *Ma. annulifera*, *Ma. annulata*, *Ma. bonneae*, *Ma. dives* and *Ma. indiana* are vectors of brugian filariasis (Wharton, 1962) and *Anopheles maculates*, *An. balabacensis*, *An. dirus*, *An. letifer*, *An. campestris*, *An. sundaicus* , *An. donaldi*, *An. leucosphyrus* and *An. flavirostris* are all vectors of malaria (Rahman *et al*., 1997). As far as *Cx. quinquefasciatus* is concerned, it is also known as the potential vector of bancroftian filariasis (Vythilingam *et al*., 2005).

Knowledge of their distribution in different environments needs to be ascertained. Mosquito surveillance remains the preliminary step used in vector monitoring and control. Mosquito surveillance of larval and adult stages by different

approaches has been frequently reported in Malaysia. The population studies of mosquito vectors have been carried out by using human landing catches (Reid, 1961; Rohani *et al*., 1999; Tan *et al*., 2008). In addition to identifying the mosquito breeding sites, container surveys have also been conducted (Cheah *et al*., 2006; Chen *et al*., 2009; Nyamah *et al*., 2010). Moreover, mosquito studies using animal-baited traps have been reported in the literature (Reid, 1961; Rohani *et al*., 1999; Tan *et al*., 2008). The use of light traps in mosquito population studies have also been documented (Vythilingam *et al*., 1992; Oli *et al*., 2005). The use of ovitraps in dengue vector surveillance has been the focus of many studies in recent years (Chen *et al*., 2005a; 2006a; 2006b; 2009; Cheah *et al*., 2006). Larval dipping is another approach used in larval population studies in the states of Pahang and Penang (Hassan *et al*., 2010; Rohani *et al*., 2010). However, little attention is being paid to larval dipping in East Malaysia.

The distribution of mosquito larvae in relation to various habitat characteristics has not yet been fully elucidated in the Southeast Asia region. There is a lack of information regarding the breeding preferences at different locations in this region. Apart from Southeast Asia, previous studies elsewhere have reported a variable relationship between larval density and habitat characteristics (Amerasinghe *et al*., 1995; Minakawa *et al*., 1999; Grillet, 2000; Muturi *et al*., 2008, De Little *et al*., 2009; Jacob *et al*., 2010).

Despite the importance of mosquitoes in the potential for disease transmission, little is known about their mixed infestation behavior. The co-occurrence of more than one species in a habitat implied that they are sharing the same environmental conditions. However, different species of mosquitoes might spread different kinds of mosquito-borne diseases and certain diseases can be transmitted by more than one species of mosquito (Miyagi & Toma, 2000). In Malaysia, several studies have reported co-occurrence among *Aedes* larvae (Chen *et al*., 2006b; Wan-Norafikah *et al*., 2009) and

co-occurrence between Anopheline and Culicine larvae (Rohani *et al*., 2010). However, no report has surfaced thus far pertaining to the mixed infestation behavior among *Culex* sp., *Lutzia* sp. and *Armigeres* sp. in stagnant water in residential areas in Malaysia.

The present study attempts to (1) determine the infestation rates of *Culex* (targeting *Cx. quinquefasciatus*) and other species of mosquitoes in stagnant water as part of an ongoing entomological investigation, (2) provide the first documented data on associations between the *Culex* distribution and various habitat characteristics and (3) elucidate the nature of co-occurrence among mosquito species in residential areas in all states of Peninsular Malaysia and East Malaysia. The findings of this study will be useful for vector control operations in these areas.

3.2 MATERIALS AND METHODS

3.2.1 STUDY AREAS

The larval surveillance was conducted at 20 residential areas in Peninsular Malaysia and East Malaysia from February to July 2011. The geographical description of the study sites is presented in Figure 3.1 and Table 3.1. There was no distinct wet or dry season throughout the year and rain was experienced every single month. However, seasonal rainfall variation occurred in every part of Malaysia during the northeast and northwest monsoon seasons. It has been confirmed that the sample collection during the study period was free from its influence across all states. The annual rainfall in all sites exceeds 2,000 mm. All study sites have a tropical climate with an average temperature of 32 ℃ and a relative humidity of 80% (Chen *et al*., 2006a).

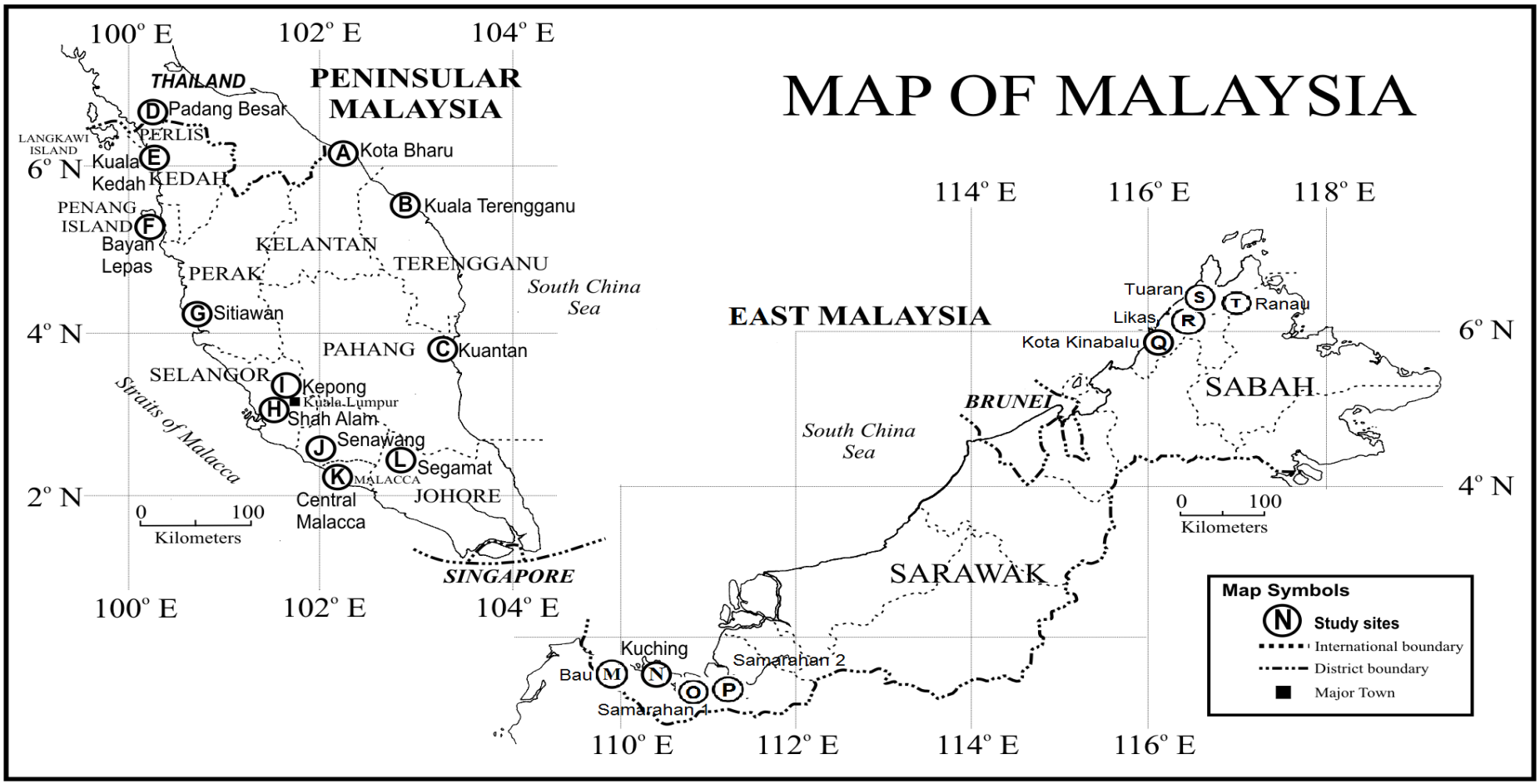

Figure 3.1 Location of study sites in Peninsular and East Malaysia.

Table 3.1 Geographical description of study sites.

Malaysia	Region	State	District	Study Site	Coordinates	Elevation (m)	Landscape
Peninsular	East Coast	Kelantan	Kota Bharu	Taman Guru	06°05’49.43”N, 102°14’06.80”E	8.53	Sub-urban
		Terengganu	Kuala Terengganu	Kg. Simpang Empat	05°15’57.73”N, 103°10’49.90”E	6.71	Rural
		Pahang	Kuantan	Taman Chenderawasih	03°48’00.40”N, 103°18’02.20”E	6.40	Sub-urban
	Northern	Perlis	Padang Besar	Taman Singgahsana	06°39’11.00”N, 100°18’54.00”E	52.43	Rural
		Kedah	Kuala Kedah	Taman Selat	06°05’02.10”N, 100°18’07.70”E	6.71	Sub-urban
		Penang	Bayan Lepas	Taman Bayan Baru	05°19’46.51”N, 100°17’24.80”E	8.84	Urban
		Perak	Sitiawan	Taman Bunga Ros	04°12’42.21”N, 100°41’42.20”E	9.75	Sub-urban
	Central	Selangor	Shah Alam	Section 17	03°02’58.28”N, 101°30’16.40”E	5.18	Urban
		Kuala Lumpur	Kepong	Kepong Baru	03°12’18.23”N, 101°38’43.60”E	50.60	Urban
	Southern	Negeri Sembilan	Senawang	Taman Marida	02°41’52.40”N, 101°59’02.44”E	79.86	Sub-urban
		Malacca	Central Malacca	Kg. Pengkalan Rama Pantai	02°12’35.77”N, 102°15’02.52”E	6.71	Rural
		Johore	Segamat	Segamat Baru	02°29’56.50”N, 102°51’12.10”E	25.60	Sub-urban
East Malaysia	West	Sarawak	Bau	Kg. Skiat Baru	01°23’53.40”N, 110°11’11.70”E	30.18	Remote
			Kuching	RPR Batu Kawa	01°31’20.40”N, 110°19’01.30”E	9.75	Sub-urban
			Samarahan 1	Kg. Rembus	01°28’59.90”N, 110°28’59.90”E	5.18	Remote
			Samarahan 2	Kg. Baru	01°29’19.40”N, 110°30’24.40”E	6.71	Remote
	East	Sabah	Kota Kinabalu	Taman Kepayan	05°56’24.96”N, 116°04’22.20”E	15.24	Sub-urban
			Likas	Taman Kingfisher	06°01’22.68”N, 116°07’22.50”E	10.06	Sub-urban
			Tuaran	Taman Kolej Tuaran	06°10’48.90”N, 116°13’41.30”E	18..59	Rural
			Ranau	Taman Delima	06°00’16.92”N, 116°48’34.90”E	444.40	Rural

3.2.2 LARVAL DIPPING METHOD

A total of 1,863 typical sources of stagnant water: drains, pools, reservoirs, canals and temporary flooded areas were surveyed for the presence of mosquito species (targeting *Cx. quinquefasciatus*) that primarily breed in stagnant water. In order to prevent water quality changes by heavy rain, all samplings were carried out at least 3 days after rain. Mosquito larvae were dipped from stagnant water by using a 330ml capacity dipper. Since there has always been a problem in relation to total number of dips taken according to the size of breeding sites, a standard dipping technique developed by Mendoza *et al*. (2008) was carried out in the present study. Standardization of the number of dips in accordance with the surface area of the water body was as follows: number of dips, water surface area (m^2): 1, 0.25; 2, 0.26–1.0; 3, 1.1–3.0; 4, 3.1–5.0; 5, 5.1–7.0; 6, 7.1–9.0 and so on. Dips were taken gently with a 2–3 min pause, to allow for the mosquito larvae to move freely in the air–water interface. Water samples were collected from sites where mosquito larvae were present. The pH, conductivity, salinity, total dissolved solids (TDS) and dissolved oxygen (DO) of the water samples were measured by using a handheld water quality meter (YSI® 556 Multi-Probe System, Yellow Springs, OH). The elevation and coordinates of each study site were recorded by using Garmin® GPS 72H (Olathe, KS).

3.2.3 SPECIES IDENTIFICATION

Field-collected larvae were placed in 500 ml plastic cups and transported to the laboratory for identification. The larvae were placed in larval rearing trays containing deionized water and provided with a fine mixture of mice chow, beef liver and milk powder in the ratio of 2:1:1 by weight. The pupae were sorted out daily and introduced

into a mosquito cage. Moribund and dead larvae were subsequently mounted for identification. The adults and larvae were identified according to illustrated keys (Rattanarithikul *et al*., 2005; 2006) and cross-referenced with the voucher specimens from the laboratory. Representative specimens from this study were used as voucher specimens and deposited in the Laboratory of Zoological and Ecological Network, University of Malaya.

3.3.4 STATISTICAL ANALYSIS

Data were analyzed to determine the following: 1) dipper index (DI), the percentage of positive dips against the total number of dips taken, 2) mean number of larvae per dip and 3) breeding index (BI), developed by Belkin (1954). Breeding index was calculated as:

$$BI = TLP / ND \times BP$$

where BI = breeding index, TLP = total number of larvae, ND = number of dips and BP = number of breeding places. The breeding place was defined as each separate microhabitat or station within a site from which 1 to 3 positive dips were obtained. Data were analyzed using the statistical program, SPSS® version 18 (Chicago, IL). Descriptive statistics were used to summarize the data for each study area. The differences between mean number of larvae per dip across all study sites were assessed by one way ANOVA. Spearman rank-order correlation was used to determine the associations between mean number of *Culex* larvae and habitat characteristics.

3.4 RESULTS

The data presented clearly indicated the study sites as natural breeding sites of mosquitoes, particularly *Culex* spp. A total of 3,117 dips were performed at 20 sampling sites, representing 4 types of residential areas: urban (n = 3), suburban (n = 8), rural (n = 6) and remote (n = 3). A total of 547 positive dips were identified, out of 3,117 dips. A total of 7,848 specimens belonging to 4 genera, namely *Culex*, *Armigeres*, *Anopheles* and *Lutzia* were collected. *Culex quinquefasciatus* (82.74%) was the dominant species, followed by *Cx. vishnui* (14.39%) and *Cx. gelidus* (2.70%). In addition, *Lu. fuscanus* (0.11%), *Ar. subalbatus* (0.05%) and *An. separatus* (0.01%) were also detected in small numbers (Table 3.2). The distribution of *Culex* spp. in four types of residential areas is represented in Figure 3.2. Overall, *Cx. quinquefasciatus* was most likely to exist in the four types of residential areas and *Cx. vishnui* was mainly found in the suburban, rural and remote areas, whereas *Cx. gelidus* was only found in rural areas. The DI and BI of mosquito larvae are presented in Table 3.3. The mean number of larvae per dip in Kota Bharu (Kelantan) was significantly higher across all study sites (F = 9.73, df = 3, 116, P = 0.000). High DI values were recorded in Kota Bharu (Kelantan), Tuaran (Sabah), Sitiawan (Perak) and Central Malacca (Malacca), accounting for 46.28, 43.48, 41.33 and 40.46%, respectively. The highest BI value, 65.39, was found in Tuaran (Sabah).

The *Culex* larvae occurred in stagnant water with pH ranging from 6.4 to 8.2; conductivity, 139.7 to 6635.2 µs/cm; salinity, 0.07 to 3.64 ppt; TDS, 0.09 to 4.27 g/l; and DO, 5.11 to 8.11 mg/l (Table 3.4). The correlation between the mean number of *Culex* larvae and habitat characteristics are presented in Figure 3.3. The Spearman rank order correlation revealed that the mean number of *Culex* larvae was positively correlated with pH (r = 0.521, P = 0.040), conductivity (r = 0.574, P = 0.022), salinity (r = 0.510, P = 0.045) and TDS (r = 0.591, P = 0.017). These positive correlations

implied that the infestation rates of *Culex* larvae increased with increasing pH, conductivity, salinity and TDS. Conversely, the elevation (r = 20.657, P = 0.005) and DO (r = 20.415, P = 0.109) were found to be negatively correlated with the mean number of *Culex* larvae. These negative correlations implied that the infestation rates of *Culex* larvae decreased with increasing elevation and DO.

The percentage of co-occurrence of mosquito larvae obtained from larval dipping method is demonstrated in Table 3.5. Eight study sites exhibited co-occurrence of mosquito larvae, namely Central Malacca (Malacca), Kota Bharu (Kelantan), Kuching (Sarawak), Ranau (Sabah), Senawang (Negeri Sembilan), Segamat (Johore), Shah Alam (Selangor) and Tuaran (Sabah). The percentage of co-occurrence according to mosquito species is presented in Table 3.6. *Culex quinquefasciatus* was able to breed simultaneously with *Cx. gelidus* (10.00-50.00%), *Lu. fuscanus* (2.94-13.33%), *Cx. vishnui* (5.00%) and *Ar. subalbatus* (1.28-3.77%). Meanwhile, *Cx. vishnui* was able to breed simultaneously with *Cx. gelidus* (20.00%) and *Lu. fuscanus* (3.33%). The ratio of mosquito species recorded from co-occurrence dips is presented in Table 3.7. Generally, *Cx. quinquefasciatus* was the dominant species in the majority of dips conducted in Central Malacca (Malacca), Kota Bharu (Kelantan), Segamat (Johore) and Shah Alam (Selangor) by 1.50-10.00 folds. However, *Cx. vishnui* was the dominant species in dips conducted in Tuaran (Sabah) and Kuching (Sarawak) by 1.67-19.00 folds. It is of interest that the drains in Tuaran (Sabah) were inhabited by *Cx. vishnui*, *Cx. gelidus* and *Cx. quinquefasciatus* but the ratio of mixed infestation of these species were low (<2).

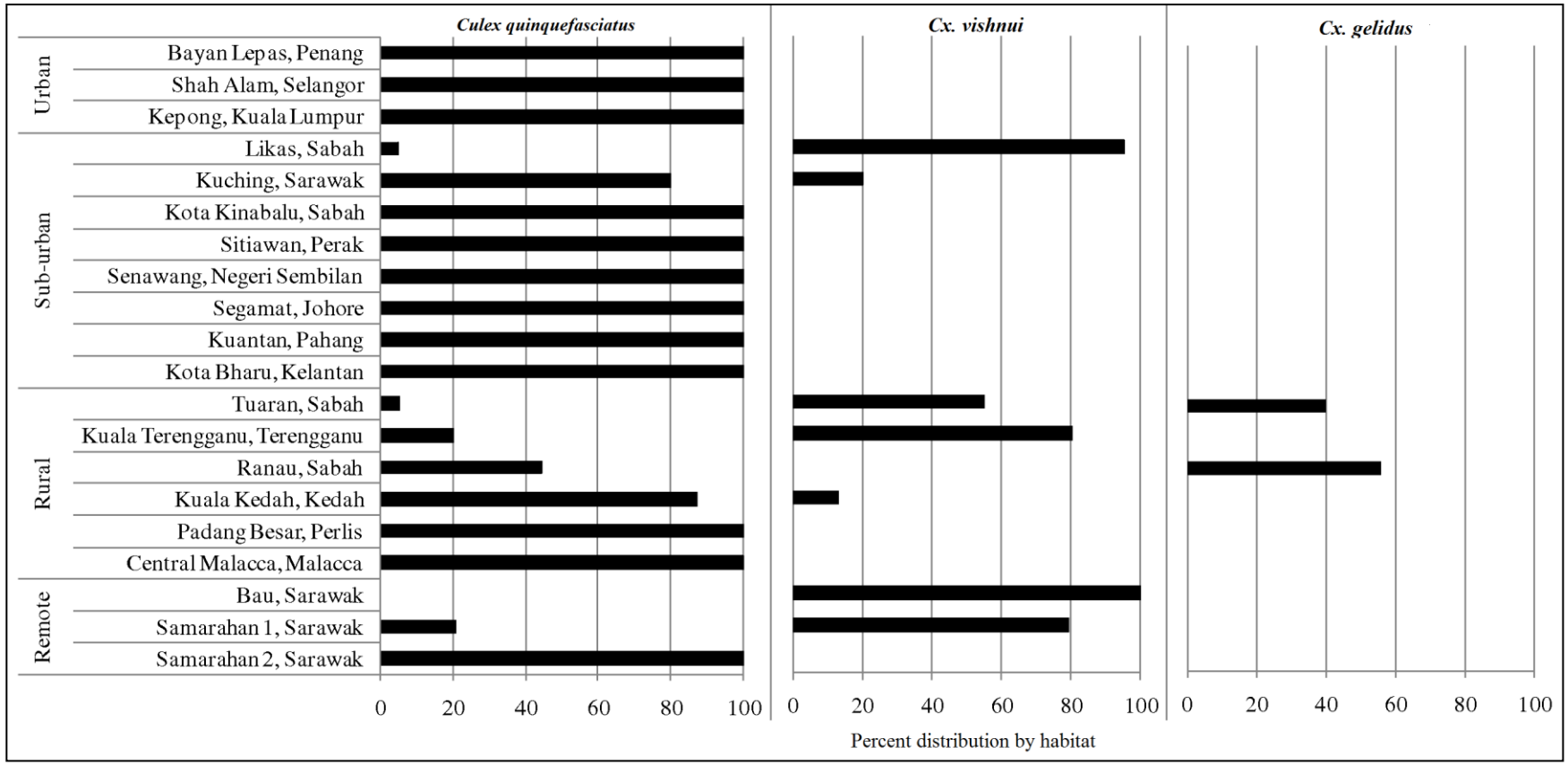

Figure 3.2 Distribution of *Culex* larvae in stagnant drainage water in four types of residential areas in Malaysia.

Table 3.2 Total number and percentage of mosquito larvae collected from various study sites.

Study Site	Total No. of Dip	*Cx.quinquefasciatus*		*Cx.vishnui*		*Cx. gelidus*		*Ar. subalbatus*		*Lu. fuscanus*		*An. separatus*		Total No. of Larvae
		n	%	n	%	n	%	n	%	n	%	n	%	
Kota Bharu, Kelantan	121	1204	99.75	0	0.00	0	0.00	0	0.00	3	0.25	0	0.00	1,207
Kuala Terengganu, Terengganu	197	138	19.80	559	80.20	0	0.00	0	0.00	0	0.00	0	0.00	697
Kuantan, Pahang	167	30	100.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	30
Padang Besar, Perlis	123	98	100.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	98
Kuala Kedah, Kedah	144	47	87.04	7	12.96	0	0.00	0	0.00	0	0.00	0	0.00	54
Bayan Lepas, Penang	212	332	100.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	332
Sitiawan, Perak	196	688	100.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	688
Shah Alam, Selangor	187	1383	99.93	0	0.00	0	0.00	0	0.00	1	0.07	0	0.00	1,384
Kepong, Kuala Lumpur	162	482	100.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	482
Senawang, Negeri Sembilan	331	155	97.48	0	0.00	0	0.00	0	0.00	4	2.52	0	0.00	159
Central Malacca, Malacca	131	445	99.55	0	0.00	0	0.00	2	0.45	0	0.00	0	0.00	447
Segamat, Johore	267	720	99.72	0	0.00	0	0.00	2	0.28	0	0.00	0	0.00	722
Kuching, Sarawak	218	374	79.75	94	20.04	0	0.00	0	0.00	1	0.21	0	0.00	469
Bau, Sarawak	86	0	0.00	7	100.00	0	0.00	0	0.00	0	0.00	0	0.00	7
Samarahan 1, Sarawak	103	6	20.69	23	79.31	0	0.00	0	0.00	0	0.00	0	0.00	29
Samarahan 2, Sarawak	113	8	100.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	8
Tuaran, Sabah	46	19	5.05	150	39.89	207	55.06	0	0.00	0	0.00	0	0.00	376
Likas, Sabah	162	15	4.93	289	95.07	0	0.00	0	0.00	0	0.00	0	0.00	304
Ranau, Sabah	53	4	40.00	0	0.00	5	50.00	0	0.00	0	0.00	1	10.00	10
Kota Kinabalu, Sabah	98	345	100.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	345
Total	**3,117**	**6,493**	**82.74**	**1,129**	**14.39**	**212**	**2.70**	**4**	**0.05**	**9**	**0.11**	**1**	**0.01**	**7,848**

Table 3.3 Dipper index, mean number of larvae per dip and breeding index obtained at various study sites.

Study Site	No.	Number of Positive	Dipper Index (DI)	Mean Number of Larvae Per Dip[1]	Breeding Index (BI)
Kota Bharu, Kelantan	121	56	46.28%	9.98±1.59	49.88
Kuala Terengganu, Terengganu	197	59	29.95%	3.54±0.54	17.69
Kuantan, Pahang	167	19	11.38%	0.18±0.05	0.72
Padang Besar, Perlis	123	11	8.94%	0.80±0.28	1.59
Kuala Kedah, Kedah	144	17	11.81%	0.36±0.10	1.88
Bayan Lepas, Penang	212	10	4.72%	1.57±0.64	1.57
Sitiawan, Perak	196	81	41.33%	3.51±0.45	21.06
Shah Alam, Selangor	187	34	18.18%	7.40±1.61	22.20
Kepong, Kuala Lumpur	162	31	19.14%	2.98±0.75	8.93
Senawang, Negeri Sembilan	331	15	4.53%	0.48±0.19	0.48
Central Malacca, Malacca	131	53	40.46%	3.41±0.63	17.06
Segamat, Johore	267	78	29.21%	2.70±0.41	13.50
Kuching, Sarawak	218	30	13.76%	1.72±0.59	15.06
Bau, Sarawak	86	5	5.81%	0.08±0.04	0.08
Samarahan 1 , Sarawak	103	10	9.71%	0.28±0.10	1.69
Samarahan 2, Sarawak	113	3	2.65%	0.07±0.04	0.14
Tuaran, Sabah	46	20	43.48%	7.99±3.05	65.39
Likas, Sabah	162	8	4.94%	1.87±0.91	5.63
Ranau, Sabah	53	2	3.77%	0.19±0.17	0.38
Kota Kinabalu, Sabah	98	5	5.10%	3.52±2.44	10.56

[1] $F = 9.73$, df = 3116, $P = 0.000$

Table 3.4 Water quality data of stagnant water samples from all study sites.

Study Site	pH	Conductivity (µs/cm)	Salinity (ppt)	TDS (g/l)	DO (mg/l)
Kota Bharu, Kelantan	7.80±0.09	364.57±58.84	0.17±0.03	0.24±0.04	7.29±0.13
Kuala Terengganu, Terengganu	7.82±0.10	6635.17±1391.64	3.64±0.80	4.27±0.90	6.23±0.09
Kuantan, Pahang	6.66±0.12	232.33±9.84	0.11±0.00	0.15±0.01	8.11±0.09
Padang Besar, Perlis	7.56±0.14	1532.00±760.08	0.81±0.41	1.02±0.51	5.11±1.73
Kuala Kedah, Kedah	7.27±0.16	617.00±18.02	0.30±0.01	0.41±0.01	6.22±0.89
Bayan Lepas, Penang	7.17±0.11	277.40±7.83	0.11±0.03	0.15±0.04	7.86±0.05
Sitiawan, Perak	7.50±0.03	362.50±3.23	0.18±0.01	0.24±0.00	5.98±0.23
Shah Alam, Selangor	7.90±0.11	337.00±30.53	0.16±0.02	0.23±0.02	7.90±0.16
Kepong, Kuala Lumpur	7.79±0.07	602.50±62.06	0.30±0.03	0.40±0.04	7.72±0.36
Senawang, Negeri Sembilan	8.22±0.09	569.50±54.48	0.28±0.03	0.37±0.04	7.48±0.22
Central Malacca, Malacca	6.92±0.10	589.00±55.65	0.29±0.03	0.39±0.04	6.42±0.55
Segamat, Johore	6.42±0.34	304.67±22.20	0.15±0.01	0.20±0.02	7.63±0.28
Kuching, Sarawak	7.12±0.06	365.20±66.75	0.18±0.03	0.24±0.04	6.50±0.51
Bau, Sarawak	7.41±0.02	192.00±5.51	0.09±0.00	0.13±0.00	7.57±0.28
Samarahan 1, Sarawak	7.34±0.06	195.00±10.82	0.09±0.01	0.13±0.01	6.52±0.40
Samarahan 2, Sarawak	7.21±0.04	690.50±5.50	0.33±0.01	0.45±0.01	6.81±0.13
Tuaran, Sabah	6.79±0.02	139.67±7.94	0.07±0.00	0.09±0.01	7.75±0.17
Likas, Sabah	6.54±0.09	334.67±79.41	0.16±0.04	0.22±0.05	5.59±0.59
Ranau, Sabah	6.81±0.10	338.00±133.00	0.17±0.07	0.23±0.09	7.47±0.07
Kota Kinabalu, Sabah	6.63±0.22	340.40±32.88	0.17±0.02	0.23±0.02	6.61±0.68

Table 3.5 Percentage of co-occurrence of mosquito larvae in residential areas.

Study Site	Total Number of Dip Conducted	Total Number of Positive Dip		Number of Co-occurrence Found in Positive Dip	
		n	%	n	%
Kota Bharu, Kelantan	121	56	46.28	2	3.57
Kuala Terengganu, Terengganu	197	59	29.95	0	0.00
Kuantan, Pahang	167	19	11.38	0	0.00
Padang Besar, Perlis	123	11	8.94	0	0.00
Kuala Kedah, Kedah	144	17	11.81	0	0.00
Bayan Lepas, Penang	212	10	4.72	0	0.00
Sitiawan, Perak	196	81	41.33	0	0.00
Shah Alam, Selangor	187	34	18.18	1	2.94
Kepong, Kuala Lumpur	162	31	19.14	0	0.00
Senawang, Negeri Sembilan	331	15	4.53	2	13.33
Central Malacca, Malacca	131	53	40.46	2	3.77
Segamat, Johore	267	78	29.21	1	1.28
Kuching, Sarawak	218	30	13.76	1	3.33
Bau, Sarawak	86	5	5.81	0	0.00
Samarahan 1, Sarawak	103	10	9.71	0	0.00
Samarahan 2, Sarawak	113	3	2.65	0	0.00
Tuaran, Sabah	46	20	43.48	7	35.00
Likas, Sabah	162	8	4.94	0	0.00
Ranau, Sabah	53	2	3.77	1	50.00
Kota Kinabalu, Sabah	98	5	5.1	0	0.00
Total	**3117**	**547**	**17.55**	**17**	**3.11**

Table 3.6 Percentage of co-occurrence according to mosquito species.

Study Site	Total No. of Positive	No. of Dip																					
		CQ		CV		CG		AS		LF		AN		CQ+LF		CQ+AS		CQ+CV		CQ+CG		CV+LF		CV+CG	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Kota Bharu, Kelantan	56	54	96.43	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	2	3.57	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
Shah Alam, Selangor	34	33	97.06	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	1	2.94	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
Senawang, Negeri Sembilan	15	13	86.67	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	2	13.33	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
Central Malacca, Malacca	53	51	96.23	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	2	3.77	0	0.00	0	0.00	0	0.00	0	0.00
Segamat, Johore	78	77	98.72	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	1	1.28	0	0.00	0	0.00	0	0.00	0	0.00
Kuching, Sarawak	30	14	46.67	15	50.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	1	3.33	0	0.00
Tuaran, Sabah	20	2	10.00	8	40.00	3	15.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	1	5.00	2	10.00	0	0.00	4	20.00
Ranau, Sabah	2	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	1	50.00	0	0.00	0	0.00	0	0.00	1	50.00	0	0.00	0	0.00
Total	**288**	**244**	**84.71**	**23**	**7.99**	**3**	**1.04**	**0**	**0.00**	**0**	**0.00**	**1**	**0.35**	**5**	**1.74**	**3**	**1.04**	**1**	**0.35**	**3**	**1.04**	**1**	**0.35**	**4**	**1.39**

CQ = *Cx. quinquefasciatus*, CV = *Cx. vishnui*, CG = *Cx. gelidus*, AS = *Ar. subalbatus*, LF = *Lu. fuscanus*, AN = *An.separatus*

Table 3.7 Ratio of mosquito species recorded from co-occurrence dips.

Study Site	CQ : LF	CQ : AS	CQ : CV	CQ : CG	CV : LF	CV : CG
Kota Bharu, Kelantan	3.33 : 1.00	0	0	0	0	0
Shah Alam, Selangor	10.00 : 1.00	0	0	0	0	0
Senawang, Negeri Sembilan	3.25 : 1.00	0	0	0	0	0
Central Malacca, Malacca	0	3.50 : 1.00	0	0	0	0
Segamat, Johore	0	1.50 : 1.00	0	0	0	0
Kuching, Sarawak	0	0	0	0	19.00 : 1.00	0
Tuaran, Sabah	0	0	1.00 : 1.67	1.22 : 1.00	0	1.00 : 1.51
Ranau, Sabah	0	0	0	1.00 : 1.25	0	0

CQ = *Cx. quinquefasciatus*, CV = *Cx. vishnui*, CG = *Cx. gelidus*, AS = *Ar. subalbatus*, LF = *Lu. fuscanus*

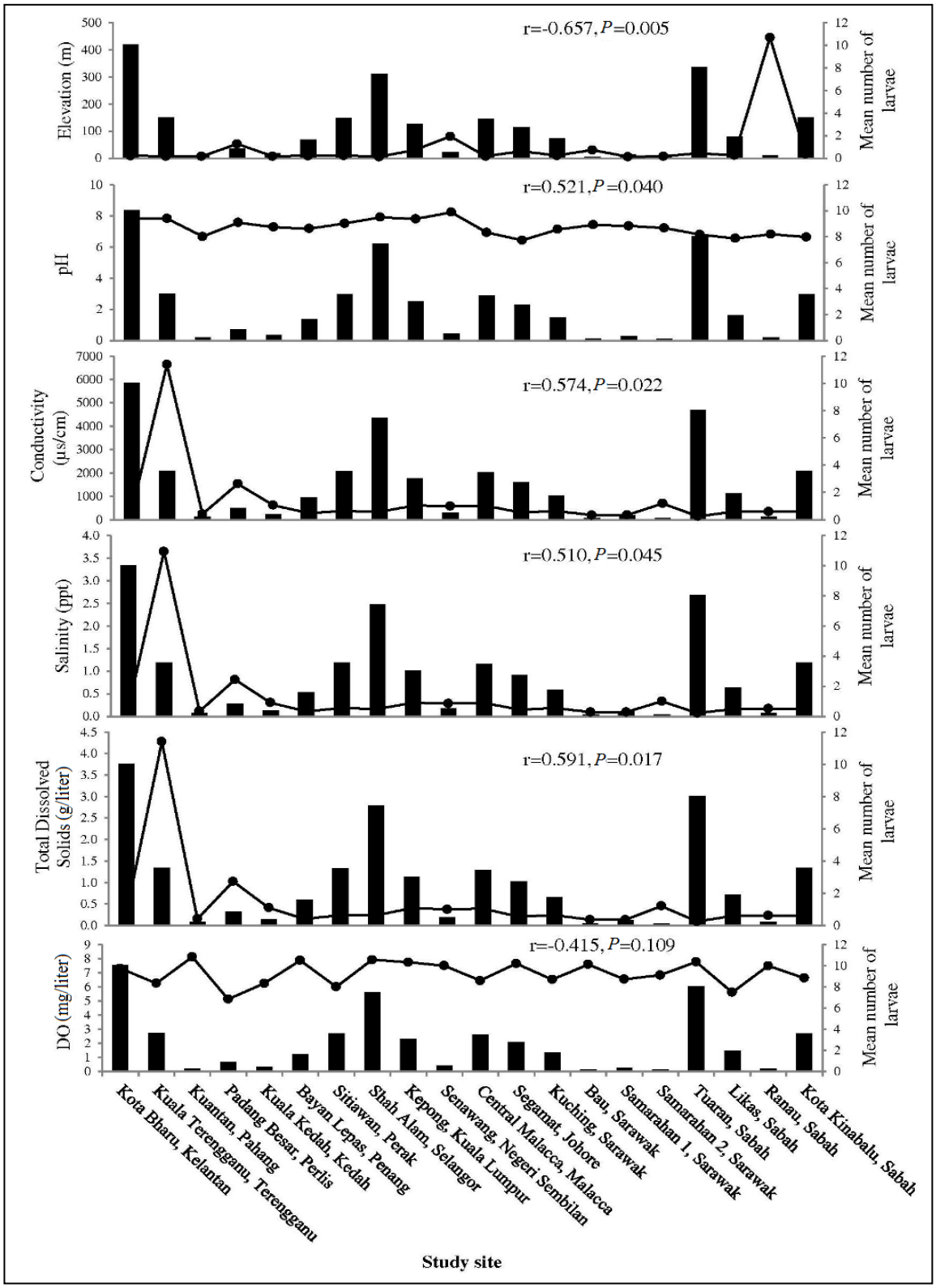

Figure 3.3 Correlation between mean number of *Culex* larvae and various habitat characteristics. Bars represent mean number of *Culex* larvae; lines represent habitat characteristics.

3.5 DISCUSSION

Based on the entomological surveys conducted in the current study, *Culex* mosquitoes appeared to be the most abundant species found across all study sites, hence confirming that stagnant water from the residential areas provided suitable larval sites for *Culex* mosquitoes. This is in agreement with the fact that *Culex* mosquitoes are most likely to lay eggs in stagnant polluted water and their breeding sites are normally near adult feeding areas (Yap *et al*., 2000a). The results of this study demonstrated that the study sites from Kota Bharu (Kelantan), Sitiawan (Perak), Shah Alam (Selangor) and Tuaran (Sabah) were dominated by *Culex* mosquitoes by exhibiting the high BI values. It was suggested that environmental conditions in these study sites favored the infestation by *Culex* mosquitoes. Vector control operations should target these study sites, as high frequency of mosquitoes will increase the risk of disease transmission. In contrast, the study sites from Kuantan (Pahang), Senawang (Negeri Sembilan), Bau (Sarawak), Samarahan 2 (Sarawak) and Ranau (Sabah) exhibited low BI values. In the current study, the number of aquatic predators has not been quantified. Considering that the majority of the sampled habitats in suburban, rural and remote areas had dragonfly nymphs, water beetles, fish and tadpoles, one of the possible reasons for the low BI values may be attributed to the presence of these aquatic predators.

The findings of this study also indicate that *Cx. quinquefasciatus* was the most widespread species and well-distributed in urban, suburban, rural and remote areas. It is a cosmopolitan mosquito species distributed in a wide range of larval habitats (Muturi *et al*., 2007a) and is the most common domestic species in urban, suburban and rural areas, where 53.2–62.7% were reported to be anthropophilic (Reuben, 1992). A wide range of distribution of *Cx. quinquefasciatus* has also been documented in Thailand (Kitvatanachai *et al*., 2005) and India (Kaliwal *et al*., 2010). Meanwhile, the distribution

of *Cx. vishnui* from the study sites supports the common belief that this species is mainly found in the rural areas. However, *Cx. vishnui* were also found in the suburban areas of East Malaysia, suggesting that its geographical distribution may contribute to the occurrence of this species in the suburban areas since East Malaysia is mostly surrounded by lowland rainforests and mountain rainforest. *Culex gelidus* mainly occurs in the rural areas, paddy fields, cultivated areas and pig farms (Tham, 2000). The presence of *Cx. gelidus* in the rural areas of Tuaran (Sabah) may be due to intensive pig farming activities in various localities in this area. The pig farms in the Tuaran area not only provide suitable potential breeding sites, but also a blood source for *Cx. gelidus*. This is supported by the observation of Miyagi & Toma (2000) who reported that *Cx. gelidus* preferred to feed on pigs, compared to humans.

Besides the presence of *Culex* mosquitoes, a relatively low number of *Lu. fuscanus, An. separates* and *Ar. subalbatus* were also detected in stagnant water. It has been reported that *Lu. fuscanus*, *An. separatus* and *Ar. subalbatus* were commonly found in artificial containers (Chow, 1950), swamp areas (Wharton *et al*., 1963) and tree holes (Lien, 1962), respectively. It was suggested that these species were seeking potential breeding sites as a result of environment adaptation.

A significant negative correlation between the mean number of *Culex* larvae and elevation of larval habitat that was noted in the present study corroborated the study of Jacob *et al*., (2010), where a statistically significant inverse linear relationship between total sampled *Culex* mosquitoes and elevation has been reported. Likewise, De Little *et al*. (2009) also reported *Aedes* density correlated negatively with elevation.

With regard to water quality assessment, few studies have reported on the relationship between the density of mosquitoes and the physiochemical characteristic of water. Different environmental factors in different locations demonstrated variable results. Minakawa *et al*. (1999) found that culicine larvae exhibited significant

association with pH and Muturi *et al*. (2008) reported that *Culex* larvae were positively associated with DO and TDS. In addition, Grillet (2000) reported that the salinity and DO were associated with the spatial distribution of *Anopheles* mosquitoes. Inversely, DO was found to be negatively correlated with the mean number of *Culex* larvae in the current study, which is in agreement with the previous work by Amerasinghe *et al*. (1995). However, no significant association between the occurrence of mosquito larvae and habitat variables has been documented. It is possible that larval density may be influenced by other habitat characteristics with each contributing some effects or it may be that certain crucial factors have not yet been identified throughout the study sites (Minakawa *et al*., 1999).

The pH of habitat water ranged from 6.4 to 8.2, revealing that mosquitoes could be found in mildly acidic and alkaline environments. The highest levels of conductivity, TDS and salinity were recorded from residential areas in Kuala Terengganu (Terengganu), which is surrounded by the sea and periodically receives inflow of seawater, suggesting that salinity tolerance of mosquito larvae occurred in this area as moderate numbers of mosquito larvae, as well as DI and BI values were recorded.

The co-occurrence of mosquito species regardless of their distribution frequency might be caused by several factors. Interspecific competition between species was the obvious hypothesis tested and has been studied intensively (Braks *et al*., 2004; Costanzo *et al*., 2005; Paaijmansa *et al*., 2009). However, several studies have failed to document clear evidence for interspecific competition (Chan *et al*., 1971; Reiskind & Wilson, 2008). It has been suggested that mixed infestation between species might be caused by temporal and spatial variation, rapid and extensive urbanization, difference in fecundity between species and difference in life cycle duration between species (Chan *et al*., 1971; Leisnham & Juliano, 2009).

It is not surprising to note that *Cx. quinquefasciatus* was able to breed simultaneously with another four species of mosquito in this study as their co-occurrence with another mosquito species have been well-documented around the world. Mixed infestation between *Cx. quinquefasciatus* and *Aedes* mosquitoes has been reported from Malaysia (Chen *et al*., 2006b) and Brazil (Tubaki *et al*., 2010). Inversely, co-occurrence of *Cx. quinquefasciatus* with *Cx. nigripalpus* in Florida (Hribar, 2007) and *Cx. dolosus affinis* in Brazil (Tubaki *et al*., 2010) has also been elucidated. In Kenya, *Cx. quinquefasciatus* also co-occur with *Anopheles gambiae* (Muturi *et al*., 2007b) and *An. arabiensis* (Muturi *et al*., 2008). In the present study, *Cx. vishnui* was found to be able to breed simultaneously with *Cx. quinquefasciatus*, *Cx. gelidus* and *Lu. fuscanus*. It has been reported that *Cx. vishnui* was also co-occur with *Cx. brevipalpis* and *Cx. vishnui* complex in India and Southeast Asia regions, respectively (Sirivanakarn, 1975; Devi & Jauhari, 2007). The finding of this study demonstrated that *Ar. subalbatus* only co-occur with *Cx. quinquefasciatus*. However, previous study has pointed out that *Ar. subalbatus* was also able to breed simultaneously with a large group of mosquitoes (i.e., *Ae. krombeini*, *Ae. albolpictus*, *Cx. uniformis*, *An. elegans*, *Toxorhynchites splendens* and *Tripteroides aranoides*) in Sri Lanka (Amerasinghe, 1982). Co-occurrence of *Lu. fuscanus* with *Cx. quinquefasciatus* and *Cx. vishnui* was recorded in the present study. Previous study reported that this species acts as the predator when they co-occurred with *Ae. albopictus*, *An. sinensis*, *Cx. sitiens*, *Cx. quinquefasciatus* and *Cx. vagans* in China (Jin *et al*., 2006). However, the presence of *Lu. fuscanus*, which occurred in a very low frequency in the present study, was not seemed to be the predator of *Cx. quinquefasciatus* and *Cx. vishnui*.

In conclusion, the current study has identified the potential or actual larval habitats of mosquitoes in residential areas in Malaysia and has demonstrated several correlations between the mosquito density and habitat characteristics. This study has

also provided the first documented data on the co-occurrence of mosquito larvae among *Culex* sp., *Lutzia* sp. and *Armigeres* sp. in the residential areas in Malaysia. A more comprehensive surveillance comprising both biotic and abiotic factors needs to be taken into consideration in the near future to facilitate the management of disease transmission and mosquito control.

CHAPTER 4

MOLECULAR PHYLOGEOGRAPHY OF MALAYSIAN *CULEX QUINQUEFASCIATUS* BASED ON ANALYSES OF MITOCHONDRIAL COI AND COII GENES

4.1 INTRODUCTION

Culex is the second largest genus of Southeast Asian mosquitoes and to date a total of 94 species of *Culex* have been recorded in Malaysia (Miyagi & Toma, 2000). *Culex quinquefasciatus* is the most abundant mosquito and also a major cause of nuisance biting in Malaysia (Yap *et al*., 2000a). Its significance as the potential vector of bancroftian filariasis has been acknowledged in this region (Vythilingam *et al*., 2005).

Globally, the intraspecific genetic diversity of this species has been well-characterized in India (Sharma *et al*., 2009; Sharma *et al*., 2010; Mendki *et al*., 2011) and Bangladesh (Hasan *et al*., 2009). In 2006, a worldwide genotyping of *Cx. quinquefasciatus* comprising the continent of Asia, Africa, South America, North America, Europe and Australia has been documented (Fonseca *et al*., 2006). Important key findings from this previous study included: (1) isolates of Asian *Cx. quinquefasciatus* (i.e., India, Indonesia and Japan) and East African *Cx. quinquefasciatus* (i.e., Kenya) sharing the same genetic lineage (2) high genetic diversity of *Cx. quinquefasciatus* was demonstrated in Asian and East African populations. However, in this genetic survey, the *Cx. quinquefasciatus* from Indonesia has only been appointed as the sole representative from Southeast Asia and therefore the genetic background of Southeast Asian *Cx. quinquefasciatus* could be

underestimated. Given the limited reports on the genetic diversity of *Cx. quinquefasciatus* from Southeast Asia, the genetic lineages of this species deserve research attention, including Malaysia.

Although population studies of *Cx. quinquefasciatus* by various approaches such as light trap (Oli *et al*., 2005), human landing catches (Rohani *et al*., 2008), larval surveillance (Rohani *et al*., 2010) and container surveillance (Chen *et al*., 2009) from various localities have been extensively studied in Malaysia, no report has surfaced thus far pertaining to genetic population of this mosquito. Indeed, there is a lack of information regarding their intraspecific genetic diversity, evolutionary relationship, as well as the dispersal patterns in this region particularly in Malaysia.

Studies on phylogeny and genetic diversity in mosquitoes have been based on intraspecific markers such as NADH dehydrogenase subunit 4 (ND4) gene (da Costa-da-Silva *et al*., 2005; Herrera *et al*., 2006; Ndo *et al*., 2010), NADH dehydrogenase subunit 5 (ND5) gene (Birungi & Munstermann, 2002; Mousson *et al*., 2005; Herrera *et al*., 2006; Maia *et al*., 2009), cytochrome c oxidase subunit I (COI) gene (Mousson *et al*., 2005; Beebe *et al*., 2005; Scarpassa *et al*., 2008; Walton *et al*., 2000; Mirabello & Conn, 2006), cytochrome c oxidase subunit II (COII) gene (Mukabayire *et al*., 1999; Chen *et al*., 2004; Hasan *et al*., 2008; Hasan *et al*., 2009; Ndo *et al*., 2010), cytochrome b (cytb) gene (Mukabayire *et al*., 1999; Mousson *et al*., 2005; Ndo *et al*., 2010), 16S ribosomal RNA (16S rRNA) gene (Sharma *et al*., 2010), nuclear acetylcholinesterase-2 (ace-2) gene (Hasan *et al*., 2009), ribosomal internal transcribed spacer 2 (ITS2) gene (Mukabayire *et al*., 1999; Hasan *et al*., 2009; Ndo *et al*., 2010) and D3 domain gene (Ndo *et al*., 2010).

Based on these intraspecific markers, it has been found that mitochondrial DNA is the most widely used marker for the study of molecular ecology in animal taxa (Simon *et al*., 1994; Norris, 2002; Pramual *et al*., 2005). The assessment of genetic

variation within the species has indicated that animal mitochondrial DNA is an ideal molecular marker due to its uniparental inheritance, lack of recombination and higher rate of mutation (Lowe *et al*., 2004). Specifically, the mitochondrial genes that have been reported as reliable genetic markers for population studies are COI and COII genes (Mukabayire *et al*., 1999; Walton *et al*., 2000; Chen *et al*., 2004).

The current study aims to investigate the intraspecific genetic diversity of *Cx. quinquefasciatus* inferred from COI and COII gene sequences from 11 states and a federal territory (i.e., Kuala Lumpur) in Peninsular Malaysia as well as two states in East Malaysia that were separated by the South China Sea. Preliminary data obtained from this study showed that both markers were more variable and demonstrated higher resolution, compared to 16S rRNA and ND5. Environment adaptation resulted from local vector control activities and reproductive success of a mosquito depends greatly on its genetic variability. The application of both COI and COII genes provides significant insights into the evolution and adaptation of Malaysian *Cx. quinquefasciatus*. Moreover, identification of the common ancestor offers an opportunity to elucidate its influence on the current distribution of this species in these study areas and subsequently understand its potential risks in disease transmission. Herein, this study documented the population genetic structure of *Cx. quinquefasciatus* for the first time from residential areas in Malaysia.

4.2 MATERIALS AND METHODS

4.2.1 MOSQUITO SPECIMENS

Mosquito specimens were collected by using dipping method from 14 selected residential areas across all states in Malaysia (Table 4.1). Field collected larvae and pupae were reared to adulthood for identification. The adult mosquitoes were identified according to the illustrated keys (Rattanarithikul *et al*., 2005). The adult mosquitoes were then frozen and stored at -80 ℃ prior to DNA extraction. In the present study, a total of 70 adults *Cx. quinquefasciatus* with five individual mosquitoes representing each of the 14 study sites were randomly selected from previous nationwide collection. It has been confirmed that the selected individuals were from different sources of collection to avoid bias estimation of local diversity.

Table 4.1 Geographical description of study sites in Malaysia.

Malaysia	Region	State	District	Study site	Landscape
Peninsular	East Coast	Kelantan	Kota Bharu	Taman Guru	Sub-urban
		Terengganu	Kuala Terengganu	Kg. Simpang Empat	Rural
		Pahang	Kuantan	Taman Chenderawasih	Sub-urban
	Northern	Perlis	Padang Besar	Taman Singgahsana	Rural
		Kedah	Kuala Kedah	Taman Selat	Sub-urban
		Penang	Bayan Lepas	Taman Bayan Baru	Urban
		Perak	Sitiawan	Taman Bunga Ros	Sub-urban
	Central	Selangor	Shah Alam	Section 17	Urban
		Kuala Lumpur	Kepong	Kepong Baru	Urban
	Southern	Negeri Sembilan	Senawang	Taman Marida	Sub-urban
		Malacca	Central Malacca	Kg. Pengkalan Rama Pantai	Rural
		Johore	Segamat	Segamat Baru	Sub-urban
East Malaysia	West	Sarawak	Kuching	RPR Batu Kawa	Sub-urban
	East	Sabah	Kota Kinabalu	Taman Kepayan	Sub-urban

4.2.2 DNA EXTRACTION

Prior to DNA extraction, abdomens were dissected from mosquito samples to avoid contamination. DNA was extracted from each specimen using i-genomic CTB DNA Extraction Mini Kit^{TM} (iNtRON Biotechnology, Inc, Korea). All isolation steps were performed according to the instructions of the manufacturer (Refer Appendix A).

4.2.3 POLYMERASE CHAIN REACTION (PCR)

The amplification of extracted genomic DNA was conducted using mitochondrial primers of COI from Kumar *et al*. (2007): forward primer, 5'-GGA TTT GGA AAT TGA TTA GTT CCT T -3' and reverse primer, 5'-AAA AAT TTT AAT TCC AGT TGG AAC AGC -3'; and COII from Ndo *et al*. (2010): forward primer, 5'-TCT AAT ATG GGA GAT TAG TGC-3' and reverse primer, 5'-ACT TGC TTT CAG TCA TCT AAT G-3'. The amplification of COI and COII regions was performed in a final volume of 50µl containing 5µl 10x buffer, 2.5mM of each dNTP, 10pmol of each forward and reverse primer, 1.5U *Taq* polymerase (iNtRON Biotechnology, Inc, Korea), 25-50ng genomic DNA of mosquito. PCR was carried out using Bio-rad MyCycler^{TM} Thermal Cycler Serial Number: 580BR 7200 (CA, USA). The PCR conditions of COI included an initial denaturation of 95℃ for 5min, followed by 5 cycles of 94℃ for 40s (denaturation), 45℃ for 1min (annealing) and 72℃ for 1min (extension) and 35 cycles of 94℃ for 40s (denaturation), 51℃ for 1min (annealing), 72℃ for 1min (extension) and a final extension at 72℃ for 10min. With regard to COII, the PCR conditions included an initial denaturation of 94℃ for 3min, followed by 35 cycles of 94℃ for 30s (denaturation), 55℃ for 30s (annealing), 72℃ for 45s (extension) and a final extension at 72℃ for 10min.

4.2.4 DNA PURIFICATION

The amplified fragments were electrophoresed on 2% agarose gel pre-stained with SYBR Safe™ (Invitrogen, USA). The PCR products were purified with MEGAquick-spin™ PCR & Agarose Gel DNA Extraction System (iNtRON Biotechnology, Inc, Korea) (refer Appendix B). The purified PCR products were sent to a commercial company for DNA sequencing. Samples were sequenced using BigDyeH Terminator v3.1 Sequencing Kit™ and analyzed on ABI PRISMH 377 Genetic Analyzer™.

4.2.5 DNA SEQUENCES ALIGNMENT

Data on the nucleotide sequences of the COI and COII genes of Malaysian *Cx. quinquefasciatus* have been deposited in GenBank under the accession numbers JQ716469 to JQ716608. Sequencing data were analyzed and edited using ChromasPro 1.5® (Technelysium Pty Ltd., Australia) and BioEdit 7.0.9.0.® (Hall, 1999). The partial COI and COII sequences were preliminarily aligned using the CLUSTAL X® program (Thompson *et al*., 1997) and subsequently aligned manually.

4.2.6 HAPLOTYPE NETWORK RECONSTRUCTION

4.2.6.1 MALAYSIAN *CX. QUINQUEFASCIATUS* HAPLOTYPE

The genetic diversity or haplotype network of *Cx. quinquefasciatus* was analysed using TCS 1.13® (Clement *et al*., 2000) to calculate the minimum number of mutational steps by which the sequences can be joined with > 95% confidence. The aligned COI and

COII sequences consisted of 624bp and 685bp, respectively. The multiple sequence of both COI and COII were concatenated and yielded a total length of 1309bp.

4.2.6.2 COMPARISON OF MALAYSIAN *CX. QUINQUEFASCIATUS* WITH OTHER *CX. QUINQUEFASCIATUS* FROM GENBANK (TABLE 4.6)

The genetic diversity or haplotype network of *Cx. quinquefasciatus* was analysed using TCS 1.13® (Clement *et al*., 2000) to calculate the minimum number of mutational steps by which the sequences can be joined with > 95% confidence. The length of some sequences of *Cx. quinquefasciatus* was trimmed in order to obtain equal length of alignment for comparision and the final length of the aligned COI and COII used for analysis was 434bp and 661bp, respectively. The COI and COII sequences deposited in GenBank that did not correspond with similar length or region to the sequences of Malaysian *Cx. quinquefasciatus* generated from this study were discarded.

4.2.7 GENETIC DIVERGENCE

Uncorrected (p) pairwise genetic distances were estimated using PAUP* 4.0b10® software (Swofford, 2002) for the assessment of the level of variation in the concatenated sequences of both COI and COII genes among the representative samples.

4.2.8 PHYLOGENETIC ANALYSES

Similar sets of COI and COII sequences of *Cx. quinquefasciatus* used in haplotype analysis were aligned with other sequences of *Culex* taxa obtained from GenBank and subjected to maximum likelihood (ML), maximum-parsimony (MP), bayesian inference

(BI) and neighbour-joining (NJ) analyses. ML analysis was performed by Treefinder® version October 2008 (Jobb *et al*., 2004). BI analysis was performed using MrBayes 3.1.2® (Huelsenbeck & Ronquist, 2001). The best fit nucleotide substitution model was determined using KAKUSAN® version 3 (Tanabe, 2007), which also generates input files for ML and BI. Best fit models were evaluated using the corrected Akaike Information Criterion (Akaike, 1973; Shono, 2000) for ML and the Bayesian Information Criterion (BIC) with significance determined by Chi-square analysis. The best selected model for COI was general time-reversible (GTR) model of DNA evolution with a gamma shape parameter (G); while the best selected model for COII was J1 model with a gamma shape parameter (G). ML analysis was performed with 1000 bootstrap replicates. Two parallel runs were performed in MrBayes analysis using four chains of Markov chain Monte Carlo (MCMC). Four million Markov chain Monte Carlo (MCMC) generations were run, with convergence diagnostics calculated every 1000^{th} generation for the monitoring of the stabilization of log likelihood scores. Trees in each chain were sampled every 100^{th} generation. The likelihood scores were stabilized at 650,000 generations for COI and 550,000 generations for COII. A 50% majority rule consensus tree was generated from the sampled trees after discarding the first 20%. MP and NJ analyses were performed using PAUP* 4.0b10® (Swofford, 2002). The MP tree was constructed using the heuristic search option, 100 random sequences additions, tree bisection reconnection (TBR) branch swapping and unordered and unweighted characters. Bootstrap percentage (BP) was computed with 1000 replications. NJ bootstrap values were estimated using 1000 replicates with Kimura's two-parameter model of substitution (K2P distance) evolution model. *Ae. albopictus* (HQ398901) and *Ae. albopictus* (HQ398974) were used as outgroup for phylogenetic trees construction of COI and COII, respectively.

4.3 RESULTS

4.3.1 HAPLOTYPE NETWORK RECONSTRUCTION

4.3.1.1 MALAYSIAN *CX. QUINQUEFASCIATUS* HAPLOTYPE

A total of 70 adults *Cx. quinquefasciatus* consisting of five individual mosquitoes representing each of the 14 study sites were used for the study of intraspecific genetic diversity based on COI and COII genes. A statistical parsimony network of 70 taxa aligned as 624 characters of the COI gene revealed three haplotypes (A1-A3) (Table 4.2, Table 4.3 and Figure 4.1) while 685 characters of the COII gene revealed four haplotypes (B1-B4) (Table 4.2, Table 4.4 and Figure 4.1).

For concatenated sequences, a total of 1309 characters of both COI and COII genes revealed seven haplotypes (AB1-AB7) (Table 4.5). Results indicated that haplotype AB1 was the common ancestor and the most widespread haplotype based on its prevalence in Malaysia. On the other hand, two haplotypes (AB4 and AB6) were discovered in Kuantan (Pahang) with the absence of the common ancestor (AB1) (Figure 4.2 and Figure 4.3). There was a substitution of guanine to adenine at positions 95, 527, 731 and 770 for haplotype AB6 from a majority of the localities, AB7 from Kota Kinabalu (Sabah), AB2 from Bayan Lepas (Penang) and AB4 from Kuantan (Pahang), respectively. Haplotype AB3 from Senawang (Negeri Sembilan) consisted of two base changes where a guanine was substituted by adenine at positions 95 and 731. A substitution of adenine to guanine at position 1033 was observed in haplotype AB5 from Shah Alam (Selangor) and Kepong (Kuala Lumpur).

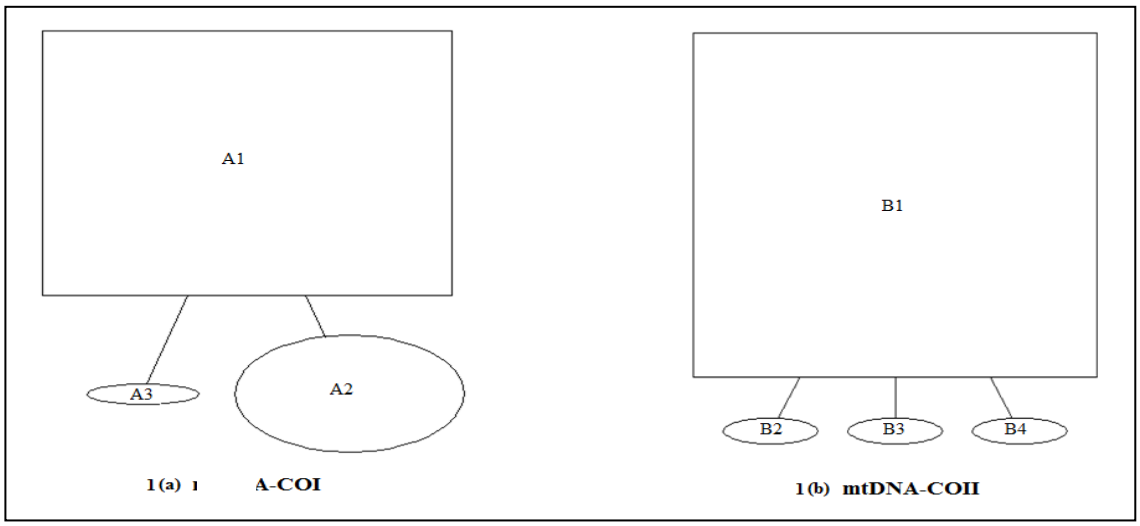

Figure 4.1 Statistical parsimony networks for COI and COII haplotypes of *Cx. quinquefasciatus* in Malaysia. Lines represent parsimonious connections between haplotypes with a probability higher than 95%, with each representing one mutational step. The size of square or oval corresponds to the haplotype frequency. Haplotype A1 and B1 were inferred as the hypothetical ancestral haplotype, respectively.

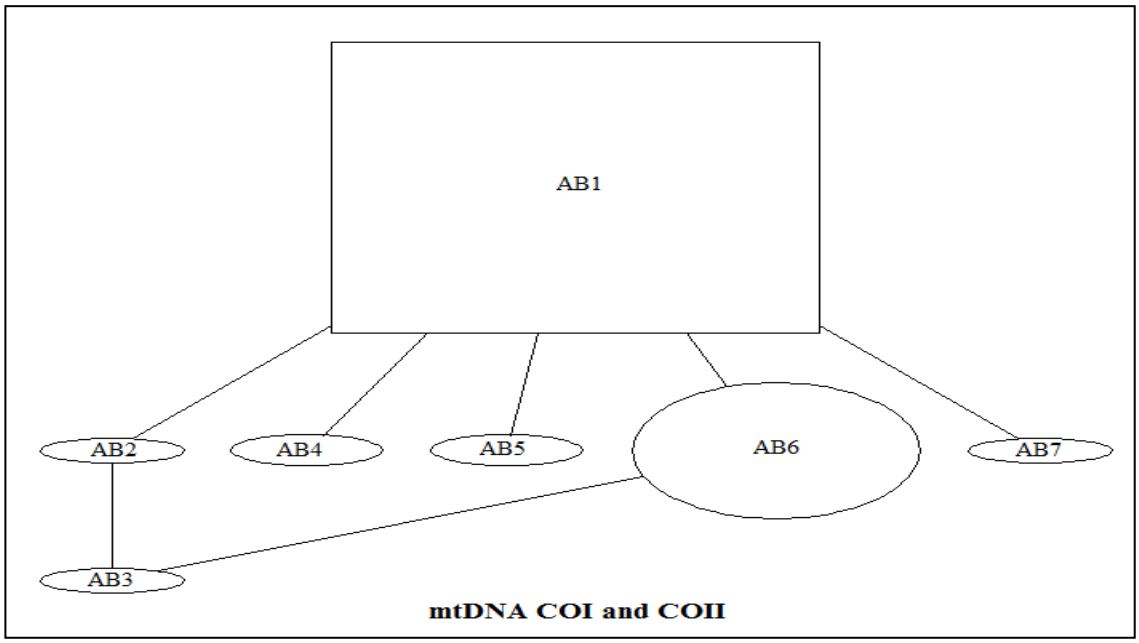

Figure 4.2 Statistical parsimony networks for concatenated sequence of COI and COII haplotypes of *Cx. quinquefasciatus* in Malaysia. Lines represent parsimonious connections between haplotypes with a probability higher than 95%, with each representing one mutational step. The size of square or oval corresponds to the haplotype frequency. Haplotype AB1 was inferred as the hypothetical ancestral haplotype.

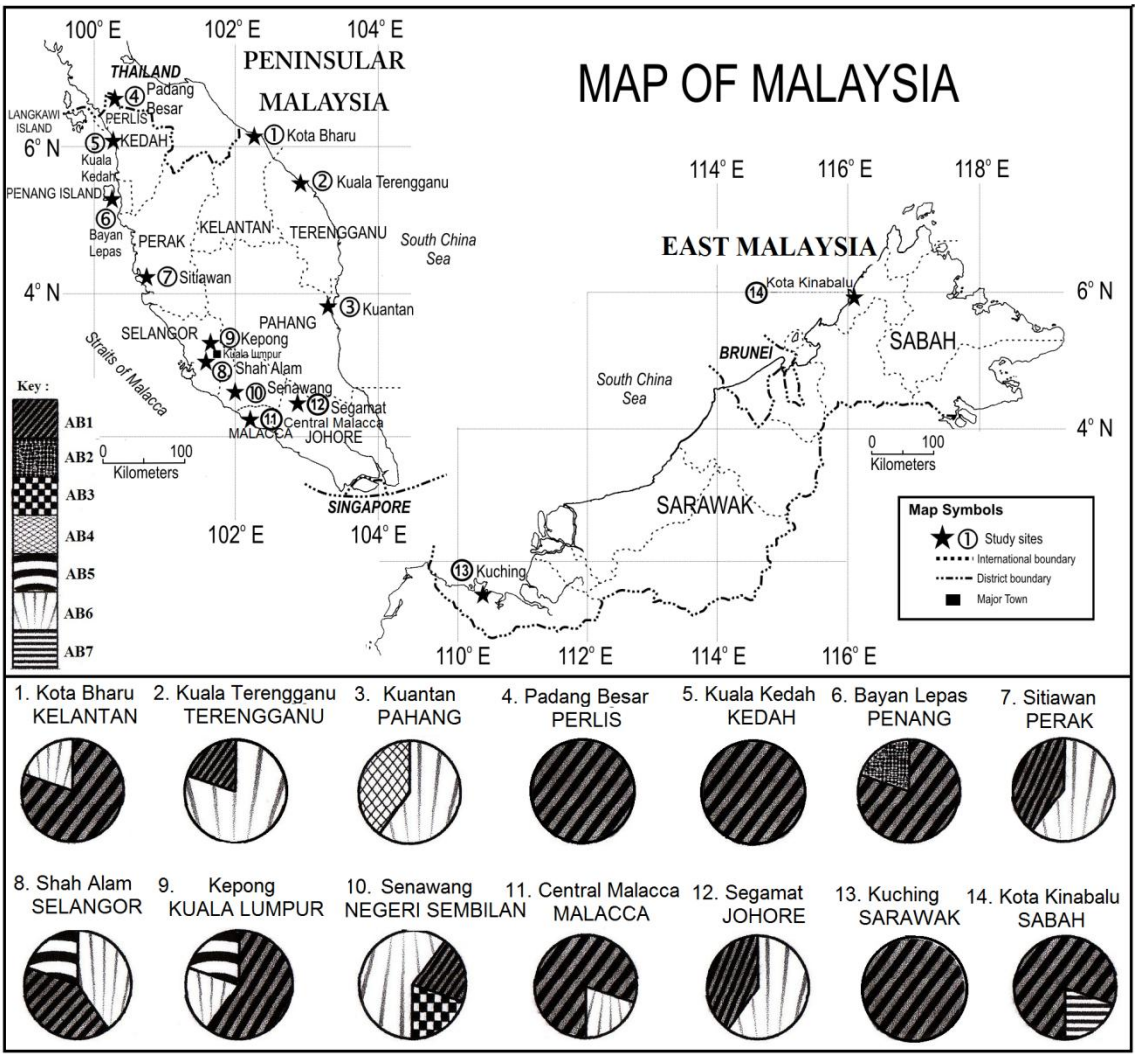

Figure 4.3 Haplotype distribution (AB1-AB7) of concatenated sequences of COI and COII for *Cx. quinquefasciatus* in Malaysia.

Table 4.2 Haplotype of *Cx. quinquefasciatus* inferred from COI and COII genes.

Study Site	n	ID Number	COI Haplotype	GenBank Accession Number	COII Haplotype	GenBank Accession Number
Kota Bharu	5	ISBUM 001	A1	JQ716488	B1	JQ716577
(Kelantan)		ISBUM 002	A1	JQ716487	B1	JQ716572
		ISBUM 003	A1	JQ716492	B1	JQ716581
		ISBUM 004	A1	JQ716507	B1	JQ716605
		ISBUM 005	A2	JQ716526	B1	JQ716559
Kuala Terengganu	5	ISBUM 006	A2	JQ716489	B1	JQ716546
(Terengganu)		ISBUM 007	A2	JQ716535	B1	JQ716574
		ISBUM 008	A1	JQ716518	B1	JQ716586
		ISBUM 009	A2	JQ716510	B1	JQ716594
		ISBUM 010	A2	JQ716506	B1	JQ716596
Kuantan	5	ISBUM 011	A2	JQ716481	B1	JQ716545
(Pahang)		ISBUM 012	A1	JQ716476	B3	JQ716548
		ISBUM 013	A2	JQ716485	B1	JQ716551
		ISBUM 014	A2	JQ716504	B1	JQ716582
		ISBUM 015	A1	JQ716529	B3	JQ716585
Padang Besar	5	ISBUM 016	A1	JQ716534	B1	JQ716604
(Perlis)		ISBUM 017	A1	JQ716469	B1	JQ716601
		ISBUM 018	A1	JQ716499	B1	JQ716561
		ISBUM 019	A1	JQ716512	B1	JQ716584
		ISBUM 020	A1	JQ716517	B1	JQ716566
Kuala Kedah	5	ISBUM 021	A1	JQ716536	B1	JQ716580
(Kedah)		ISBUM 022	A1	JQ716537	B1	JQ716541
		ISBUM 023	A1	JQ716483	B1	JQ716575
		ISBUM 024	A1	JQ716524	B1	JQ716602
		ISBUM 025	A1	JQ716532	B1	JQ716593
Bayan Lepas	5	ISBUM 026	A1	JQ716478	B1	JQ716600
(Penang)		ISBUM 027	A1	JQ716486	B4	JQ716564
		ISBUM 028	A1	JQ716484	B1	JQ716557
		ISBUM 029	A1	JQ716474	B1	JQ716608
		ISBUM 030	A1	JQ716508	B1	JQ716543
Sitiawan	5	ISBUM 031	A2	JQ716502	B1	JQ716549
(Perak)		ISBUM 032	A1	JQ716516	B1	JQ716542
		ISBUM 033	A1	JQ716528	B1	JQ716544
		ISBUM 034	A2	JQ716533	B1	JQ716571
		ISBUM 035	A2	JQ716527	B1	JQ716583
Shah Alam	5	ISBUM 036	A1	JQ716511	B1	JQ716589
(Selangor)		ISBUM 037	A2	JQ716503	B1	JQ716599
		ISBUM 038	A1	JQ716475	B2	JQ716590

Table 4.2 (continued)

Study Site	n	ID Number	COI Haplotype	GenBank Accession Number	COII Haplotype	GenBank Accession Number
		ISBUM 039	A2	JQ716505	B1	JQ716588
		ISBUM 040	A1	JQ716472	B1	JQ716554
Kepong	5	ISBUM 041	A1	JQ716509	B2	JQ716547
(Kuala Lumpur)		ISBUM 042	A2	JQ716500	B1	JQ716607
		ISBUM 043	A1	JQ716530	B1	JQ716560
		ISBUM 044	A1	JQ716479	B1	JQ716565
		ISBUM 045	A1	JQ716477	B1	JQ716573
Senawang	5	ISBUM 046	A2	JQ716501	B4	JQ716555
(Negeri Sembilan)		ISBUM 047	A2	JQ716490	B1	JQ716579
		ISBUM 048	A1	JQ716482	B1	JQ716578
		ISBUM 049	A2	JQ716514	B1	JQ716603
		ISBUM 050	A2	JQ716515	B1	JQ716567
Central Malacca	5	ISBUM 051	A1	JQ716497	B1	JQ716558
(Malacca)		ISBUM 052	A1	JQ716519	B1	JQ716553
		ISBUM 053	A1	JQ716493	B1	JQ716563
		ISBUM 054	A1	JQ716523	B1	JQ716569
		ISBUM 055	A2	JQ716522	B1	JQ716587
Segamat	5	ISBUM 056	A1	JQ716513	B1	JQ716552
(Johore)		ISBUM 057	A1	JQ716471	B1	JQ716540
		ISBUM 058	A2	JQ716480	B1	JQ716550
		ISBUM 059	A1	JQ716470	B1	JQ716570
		ISBUM 060	A1	JQ716473	B1	JQ716568
Kuching	5	ISBUM 061	A1	JQ716496	B1	JQ716591
(Sarawak)		ISBUM 062	A1	JQ716494	B1	JQ716576
		ISBUM 063	A1	JQ716498	B1	JQ716562
		ISBUM 064	A1	JQ716531	B1	JQ716597
		ISBUM 065	A1	JQ716520	B1	JQ716606
Kota Kinabalu	5	ISBUM 066	A1	JQ716495	B1	JQ716539
(Sabah)		ISBUM 067	A1	JQ716538	B1	JQ716592
		ISBUM 068	A1	JQ716491	B1	JQ716556
		ISBUM 069	A3	JQ716525	B1	JQ716595
		ISBUM 070	A1	JQ716521	B1	JQ716598

Table 4.3 Variation site in COI sequence of *Cx. quinquefasciatus* for mitochondrial haplotype from various localities in Malaysia.

Haplotype	**Origin**	**Variation Site in DNA Sequence**	
		95	527
A1	Kota Bharu, Kelantan	G	G
	Kuala Terengganu, Terengganu		
	Kuantan, Pahang		
	Padang Besar, Perlis		
	Kuala Kedah, Kedah		
	Bayan Lepas, Penang		
	Sitiawan, Perak		
	Shah Alam, Selangor		
	Kepong, Kuala Lumpur		
	Senawang, Negeri Sembilan		
	Central Malacca, Malacca		
	Segamat, Johore		
	Kuching, Sarawak		
	Kota Kinabalu, Sabah		
A2	Kota Bharu, Kelantan	A	G
	Kuala Terengganu, Terengganu		
	Kuantan, Pahang		
	Sitiawan, Perak		
	Shah Alam, Selangor		
	Kepong, Kuala Lumpur		
	Senawang, Negeri Sembilan		
	Central Malacca, Malacca		
	Segamat, Johore		
A3	Kota Kinabalu, Sabah	G	A

Table 4.4 Variation site in COII sequence of *Cx. quinquefasciatus* for mitochondrial haplotype from various localities in Malaysia.

Haplotype	**Origin**	**Variation Site in DNA Sequence**		
		107	146	409
B1	Kota Bharu, Kelantan	G	G	A
	Kuala Terengganu, Terengganu			
	Kuantan, Pahang			
	Padang Besar, Perlis			
	Kuala Kedah, Kedah			
	Bayan Lepas, Penang			
	Sitiawan, Perak			
	Shah Alam, Selangor			
	Kepong, Kuala Lumpur			
	Senawang, Negeri Sembilan			
	Central Malacca, Malacca			
	Segamat, Johore			
	Kuching, Sarawak			
	Kota Kinabalu, Sabah			
B2	Bayan Lepas, Penang	A	G	A
	Senawang, Negeri Sembilan			
B3	Kuantan, Pahang	G	A	A
B4	Shah Alam, Selangor			
	Kepong, Kuala Lumpur	G	G	G

Table 4.5 Variation site in concatenated COI and COII sequences of *Cx. quinquefasciatus* for mitochondrial haplotype from various localities in Malaysia.

Haplotype	**Origin**	**Variation Site in DNA Sequence**				
		95	527	731	770	1033
AB1	Kota Bharu, Kelantan	G	G	G	G	A
	Kuala Terengganu, Terengganu					
	Padang Besar, Perlis					
	Kuala Kedah, Kedah					
	Bayan Lepas, Penang					
	Sitiawan, Perak					
	Shah Alam, Selangor					
	Kepong, Kuala Lumpur					
	Senawang, Negeri Sembilan					
	Central Malacca, Malacca					
	Segamat, Johore					
	Kuching, Sarawak					
	Kota Kinabalu, Sabah					
AB2	Bayan Lepas, Penang	G	G	A	G	A
AB3	Senawang, Negeri Sembilan	A	G	A	G	A
AB4	Kuantan, Pahang	G	G	G	A	A
AB5	Shah Alam, Selangor	G	G	G	G	G
	Kepong, Kuala Lumpur					
AB6	Kota Bharu, Kelantan	A	G	G	G	A
	Kuala Terengganu, Terengganu					
	Kuantan, Pahang					
	Sitiawan, Perak					
	Shah Alam, Selangor					
	Kepong, Kuala Lumpur					
	Senawang, Negeri Sembilan					
	Central Malacca, Malacca					
	Segamat, Johore					
AB7	Kota Kinabalu, Sabah	G	A	G	G	A

4.3.1.2 COMPARISON OF MALAYSIAN *CX. QUINQUEFASCIATUS* WITH OTHER *CX. QUINQUEFASCIATUS* FROM GENBANK

For comparison purposes, the COI and COII sequences of *Cx. quinquefasciatus* from other countries were obtained from GenBank (Table 4.6). Four haplotypes (AA1-AA4) were revealed when COI sequences of *Cx. quinquefasciatus* from Uganda, India, Iran and Thailand were compared with Malaysian *Cx. quinquefasciatus* sequences (Table 4.7 and Figure 4.4). There was a substitution of guanine to adenine at position 95 for haplotype AA2. Haplotype AA3 from Iran with two base changes where a guanine was substituted by adenine at positions 8 and 95. Haplotype AA4 from Thailand had three base changes where an adenine was substituted by guanine at positions 257 and 386 followed by a substitution of thymine to cytosine at position 425.

On the other hand, nine haplotypes (BB1-BB9) were revealed when COII sequences of *Cx. quinquefasciatus* from Bangladesh, China, Taiwan and Thailand were compared with Malaysian *Cx. quinquefasciatus* sequences (Table 4.8 and Figure 4.4). In China 1, there was a substitution of guanine to thymine at position 22 and a substitution of thymine to cytosine at position 404, as revealed in haplotype BB2. A substitution of adenine to guanine at position 384 was revealed in haplotype BB3 from Shah Alam (Selangor) and Kepong (Kuala Lumpur). For haplotype BB4, a guanine was substituted by adenine at position 82. The haplotype BB5 from China 2 revealed three mutation changes: a guanine to adenine at position 385, a thymine to guanine at position 629 and an insertion of adenine at position 638. Besides, haplotype BB6 from China 2 also revealed two mutation changes: a guanine to adenine at position 121 and an insertion of adenine at position 638. A substitution of guanine to adenine at position 121, cytosine to adenine at position 531 and adenine to guanine at position 582, were detected in haplotype BB7 from Kuantan (Pahang), haplotype BB8 from Thailand and

haplotype BB9 from Bangladesh, respectively. Both COI and COII inferred that haplotypes AA1 and BB1 were the common ancestors and the most widespread haplotypes in a majority of the countries (Figure 4.4).

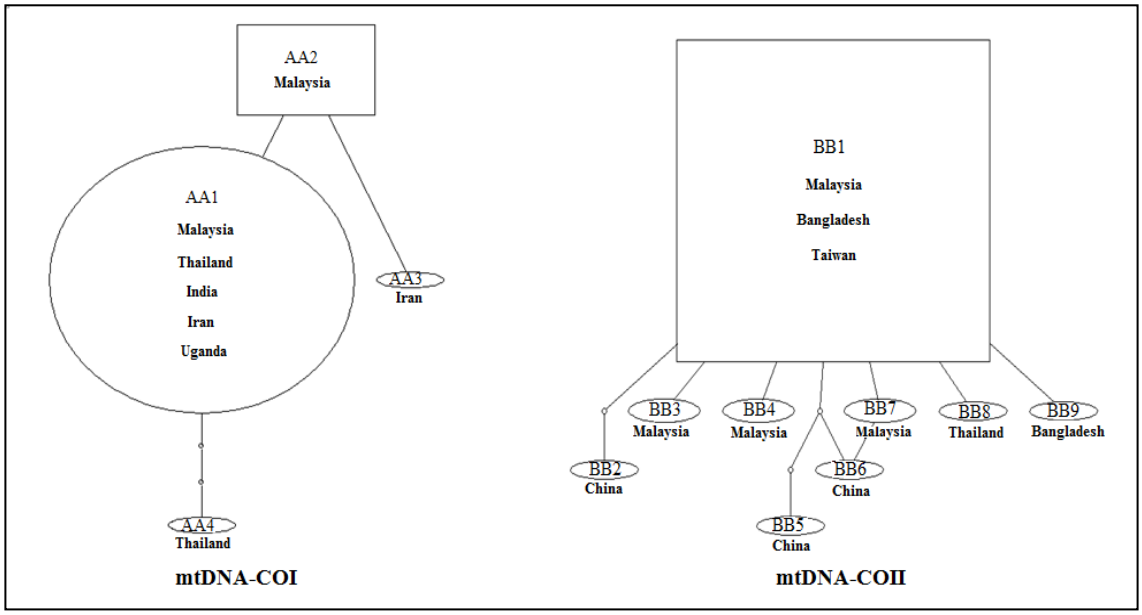

Figure 4.4 Statistical parsimony networks for COI and COII haplotypes of *Culex quinquefasciatus* in Malaysia and other countries. Lines represent parsimonious connections between haplotypes with a probability higher than 95%, with each representing one mutational step and the small circles indicate missing haplotype. The size of square or oval corresponds to the haplotype frequency. Haplotype AA1 and BB1 were inferred as the hypothetical ancestral haplotype, respectively.

Table 4.6 Haplotype of *Cx. quinquefasciatus* from other countries by COI and COII genes.

Country	n	GenBank Accession Number	Haplotype
		COI Gene	
Uganda	9	GQ165791	AA1
		GQ165792	AA1
		GQ165793	AA1
		GQ165794	AA1
		GQ165795	AA1
		GQ165796	AA1
		GQ165797	AA1
		GQ165798	AA1
		GQ165807	AA1
India 1	4	FN395201	AA1
		FN395202	AA1
		FN395204	AA1
		FN395205	AA1
India 2	1	AY729977	AA1
Iran	3	FJ210901	AA3
		FJ210909	AA1
		FJ210910	AA1
Thailand	1	HQ398883	AA4
		COII Gene	
Bangladesh	2	EU014281	BB1
		EU014282	BB9
China 2	2	AY949854	BB5
		AY949855	BB6
China 1	1	AF325716	BB2
Taiwan	1	L34351	BB1
Thailand	1	HQ398945	BB8

Table 4.7 Variation site in COI sequence of *Cx. quinquefasciatus* for mitochondrial haplotype from various localities in Malaysia and other countries.

Haplotype	Origin	Variation Site in DNA Sequence				
		8	95	257	386	425
AA1	Kota Bharu, Kelantan	G	G	A	A	T
	Kuala Terengganu, Terengganu					
	Kuantan, Pahang					
	Padang Besar, Perlis					
	Kuala Kedah, Kedah					
	Bayan Lepas, Penang					
	Sitiawan, Perak					
	Shah Alam, Selangor					
	Kepong, Kuala Lumpur					
	Senawang, Negeri Sembilan					
	Central Malacca, Malacca					
	Segamat, Johore					
	Kuching, Sarawak					
	Kota Kinabalu, Sabah					
	Uganda					
	India 1					
	India 2					
	Iran					
	Thailand					
AA2	Kota Bharu, Kelantan	G	A	A	A	T
	Kuala Terengganu, Terengganu					
	Kuantan, Pahang					
	Sitiawan, Perak					
	Shah Alam, Selangor					
	Kepong, Kuala Lumpur					
	Senawang, Negeri Sembilan					
	Central Malacca, Malacca					
	Segamat, Johore					
AA3	Iran	A	A	A	A	T
AA4	Thailand	G	G	G	G	C

Table 4.8 Variation site in COII sequence of *Cx. quinquefasciatus* for mitochondrial haplotype from various localities in Malaysia and other countries.

Haplotype	Origin	Variation Site in DNA Sequence									
		22	82	121	384	385	404	531	582	629	638
BB1	Kota Bharu, Kelantan	G	G	G	A	G	T	C	A	T	–
	Kuala Terengganu, Terengganu										
	Kuantan, Pahang										
	Padang Besar, Perlis										
	Kuala Kedah, Kedah										
	Bayan Lepas, Penang										
	Sitiawan, Perak										
	Shah Alam, Selangor										
	Kepong, Kuala Lumpur										
	Senawang, Negeri Sembilan										
	Central Malacca, Malacca										
	Segamat, Johore										
	Kuching, Sarawak										
	Kota Kinabalu, Sabah										
	Bangladesh										
	Taiwan										
BB2	China 1	T	G	G	A	G	C	C	A	T	–
BB3	Shah Alam, Selangor	G	G	G	G	G	T	C	A	T	–
	Kepong, Kuala Lumpur										
BB4	Bayan Lepas, Penang	G	A	G	A	G	T	C	A	T	–
	Senawang, Negeri Sembilan										
BB5	China 2	G	G	G	A	A	T	C	A	G	A
BB6	China 2	G	G	A	A	G	T	C	A	T	A
BB7	Kuantan, Pahang	G	G	A	A	G	T	C	A	T	–
BB8	Thailand	G	G	G	A	G	T	A	A	T	–
BB9	Bangladesh	G	G	G	A	G	T	C	G	T	–

4.3.2 GENETIC DIVERGENCE

The uncorrected '*p*' distances between different haplotype of *Cx. quinquefasciatus* based on concatenated sequences of both COI and COII genes are summarized in Tables 4.9.

Table 4.9 Uncorrected '*p*' distance matrix between Malaysian *Cx. quinquefasciatus* based on concatenated sequences of both COI and COII genes.

Haplotype	**1**	**2**	**3**	**4**	**5**	**6**
1. AB1						
2. AB2	0.00076					
3. AB3	0.00153	0.00076				
4. AB4	0.00076	0.00153	0.00229			
5. AB5	0.00076	0.00153	0.00229	0.00153		
6. AB6	0.00076	0.00153	0.00076	0.00153	0.00153	
7. AB7	0.00076	0.00153	0.00229	0.00153	0.00153	0.00153

4.3.3 PHYLOGENETIC ANALYSES

The aligned partial sequences of COI consisted of 434 sites, of which 315 characters were constant, 84 characters were parsimony informative and 35 characters were parsimony uninformative. MP analysis demonstrated a consistency index of 0.5618 and retention index of 0.5753. The aligned partial sequences of COII consisted of 662 sites; of which 514 characters were constant, 67 characters were parsimony informative and 81 characters were parsimony uninformative. MP analysis demonstrated a consistency index of 0.7149 and retention index of 0.7186.

Four phylogenetic analyses produced the same topology of the phylogenetic trees but differed in the bootstrap support values (Figure 4.5 and Figure 4.6). Only ML trees were presented for the sequences of COI and COII.

4.3.3.1 CYTOCHROME C OXIDASE SUBUNIT I (COI)

The COI ML tree comprised of two main groups (Figure 4.5). The first group consisted of *Cx. nigropunctatus* with no bootstrap support value. The second group with no bootstrap to low bootstrap support values (MP=58%, BI=51%) was further divided into two main subgroups. The first subgroup consisted of *Cx. tritaeniorhynchus*, which is the basal group and was supported with no bootstrap to high bootstrap support values (ML=54%, BI=92%, NJ=50%). The second subgroup consisted of *Cx. quinquefasciatus*, *Cx. fuscocephala*, *Cx. bitaeniorhynchus*, *Cx. gelidus* and *Cx. rubithoracis* with no bootstrap support. The second subgroup was further divided into two main clades: clade 1 and clade 2. Clade 1 consisted of *Cx. gelidus* and *Cx. rubithoracis* with no bootstrap support. Isolates of Indian and Thai *Cx. gelidus* were grouped in a monophyletic clade and supported with high to full bootstrap support values (ML=99%, MP=100%,

BI=100%, NJ=100%). Meanwhile, clade 2 was further divided into two subclades: subclade 1 which consisted of *Cx. fuscocephala* and *Cx bitaeniorhynchus*. The India and isolates of Thai *Cx. fuscocephala* were grouped in a monophyletic clade and supported with full bootstrap values (ML=100%, MP=100%, BI=100%, NJ=100%); subclade 2, consisted of two main groups: (1) *Cx. quinquefasciatus* from Uganda, India, Iran, Thailand and Malaysia (haplotype AA1 and AA4) and were supported with low bootstrap values (ML=64, MP=63, BI=68, NJ=65) and; (2) *Cx. quinquefasciatus* from Malaysia and Iran (haplotype AA2 and AA3) with no bootstrap value.

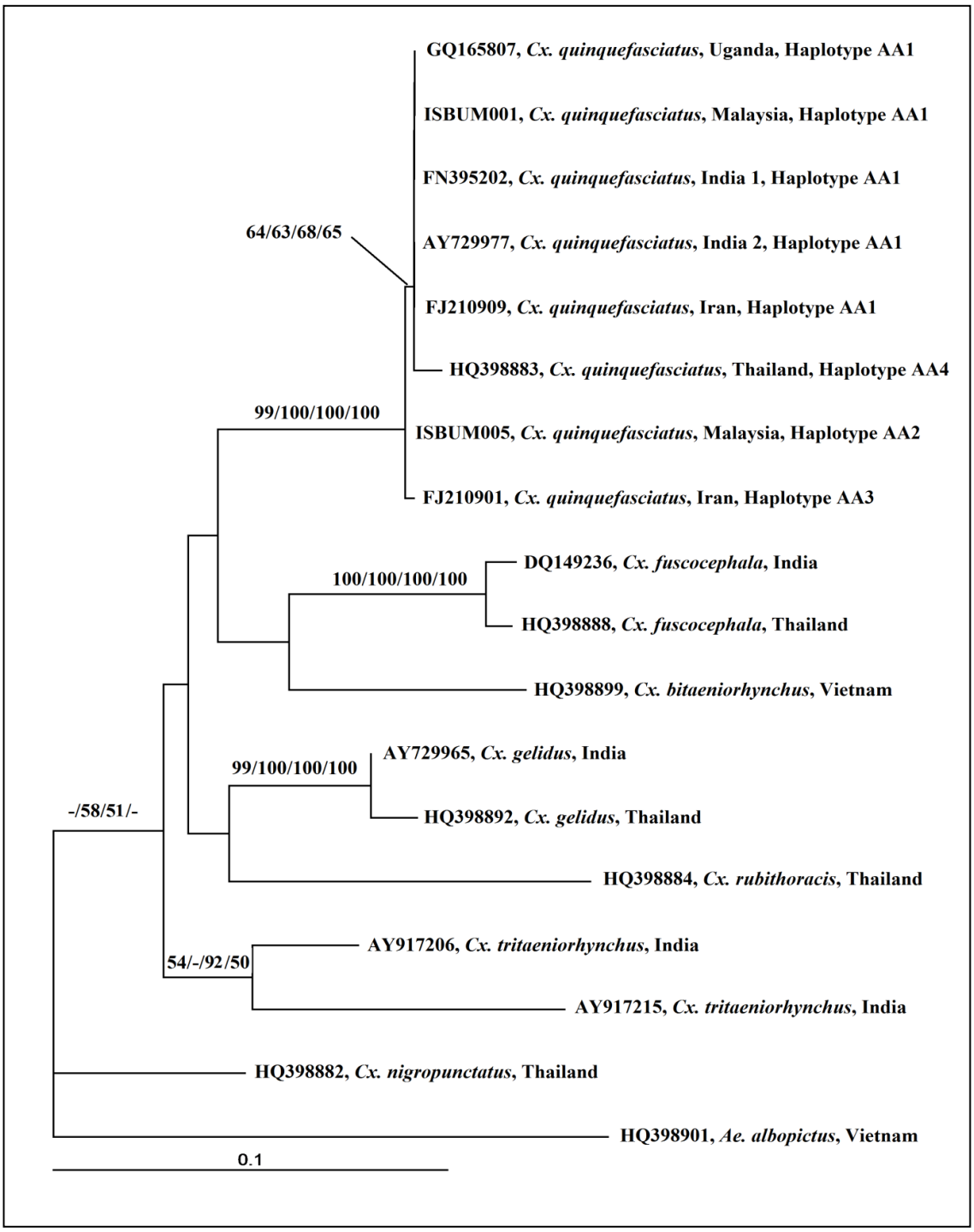

Figure 4.5 Maximum likelihood phylogeny tree of *Culex* taxa based on COI sequences. The bootstrap values (ML/MP/BI/NJ) are shown at the branches. Bar indicates substitutions per site

4.3.3.2 CYTOCHROME C OXIDASE SUBUNIT II (COII)

The COII ML tree comprised of two main groups (Figure 4.6). The first group with no boostrap support consisted of *Cx. fuscocephala, Cx. gelidus, Cx. tritaeniorhynchus* and *Cx. bitaeniorhynchus*. *Culex fuscocephala* and *Cx. gelidus* were the basal species and showed a sister relationship to *Cx. tritaeniorhynchus* and *Cx. bitaeniorhynchus* which was supported by moderate to high bootstrap support (ML=86%, MP=79%, BI=97%, NJ=73%). Isolates of *Cx. bitaeniorhynchus* from Thailand and Vietnam which grouped in a monophyletic clade was supported with high to full bootstrap values (ML=90%, MP=98%, BI=100%, NJ=100%). For another main group, it was divided into two subgroups. Subgroup 1 comprised *Cx. nigropunctatus* and *Cx. rubithoracis* with moderate to high bootstrap support values (ML=72%, MP=70%, BI=92%, NJ=72%). Subgroup 2 consisted of all the *Cx. quinquefasciatus* from various localities, supported by full bootstrap support values (ML=100%, MP=100%, BI=100%, NJ=100%). The Thai *Cx. quinquefasciatus*, haplotype BB8 was the most basal member and showed a sister relationship to the others *Cx. quinquefasciatus* which was supported with low to no bootstrap support (ML= 53%). The isolates (AY949854 and ISBUM012) from China and Malaysia (haplotype BB5 and BB7) differed from other haplotypes with low to high bootstrap support values (ML=64%, MP=63%, BI=96%, NJ=62%).

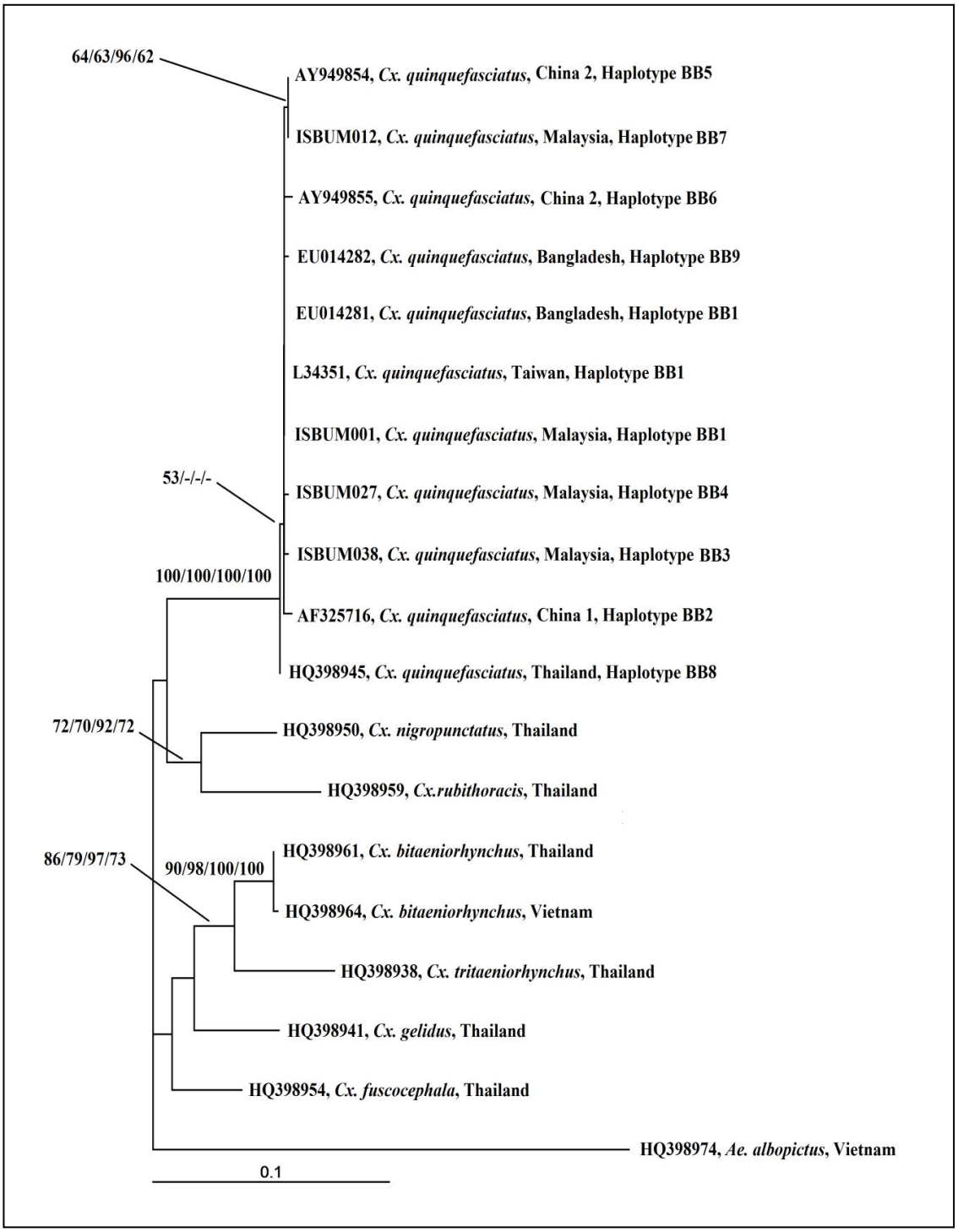

Figure 4.6 Maximum likelihood phylogeny tree of *Culex* taxa based on COII sequences. The bootstrap values (ML/MP/BI/NJ) are shown at the branches. Bar indicates substitutions per site.

4.4 DISCUSSION

Over the years, the genetic diversity of mosquitoes using COI gene (Walton *et al*., 2000; Beebe *et al*., 2005; Mousson *et al*., 2005; Mirabello & Conn, 2006; Scarpassa *et al*., 2008) and COII genes (Mukabayire *et al*., 1999; Chen *et al*., 2004; Hasan *et al*., 2008; Hasan *et al*., 2009; Ndo *et al*., 2010) has been frequently reported. In terms of variability and reliability of these markers, variable results were demonstrated from different regions. From these previous studies, both genes have been reputed as reliable and useful genetic markers in the study of Walton *et al*. (2000) and Chen *et al*. (2004), by revealing 70 haplotypes from 14 study sites (84 individuals) throughout Thailand, Myanmar and Bangladesh; 50 haplotypes from 16 study sites (76 individuals) throughout southern China and northern Vietnam, respectively. In the current study, the intraspecific genetic diversity of Malaysian *Cx. quinquefasciatus* was investigated using COI and COII genes. Across all study sites, a total of 3 haplotypes were revealed by COI gene while 4 haplotypes were revealed by COII gene. Likewise, when compared with sequences from other countries, more haplotypes were inferred from COII gene (Figure 4.4a and Figure 4.4b). The findings of this study indicated that COII gene was more variable and informative, compared with COI gene.

The results of TCS analysis indicated that haplotype AB1 was the common ancestor and the most widespread haplotype for *Cx. quinquefasciatus* due to its dispersion in Malaysia (Figure 4.2 and Figure 4.3). *Culex quinquefasciatus* from Shah Alam (Selangor), Kepong (Kuala Lumpur) and Senawang (Negeri Sembilan) demonstrated higher divergence with the identification of three different haplotypes. Inversely, the least genetic diversity was detected in Padang Besar (Perlis), Kuala Kedah (Kedah) and Kuching (Sarawak) since only one haplotype (AB1) was recorded. It was proposed that haplotype AB1 was the common ancestor of *Cx. quinquefasciatus*

and evolved over time into the various haplotypes, namely, AB2, AB3, AB4, AB5, AB6 and AB7, in order to compete with environmental changes and consequently distributed across all states in Malaysia.

The genetic distance based on concatenated sequences of both COI and COII genes ranged from 0.00076 to 0.00229. A relatively low genetic distance between Malaysian *Cx. quinquefasciatus* from various localities contrasted sharply with the Indian *Cx. quinquefasciatus*, where the highest genetic distance of 0.50117 based on 16 rRNA sequences has been recorded (Sharma *et al*., 2010). The low genetic distance between the haplotypes indicated that the genetic diversity of Malaysian *Cx. quinquefasciatus* was extremely low.

With regard to phylogenetic analyses, both COI and COII sequences of *Cx. quinquefasciatus* revealed that there were no distinct genetic lineages between Malaysia and our neighboring country (i.e., Thailand) as well as the isolates from other Asian countries (i.e., India, Iran, China, Bangladesh and Taiwan) and East Africa (i.e., Uganda). Although there are limited COI and COII sequences deposited in the GenBank and some of the sequences were discarded in order to correspond with similar length or region to the sequences of Malaysian *Cx. quinquefasciatus* generated from this study, the phylogenetic relationships comprising the isolates from Europe, America, Australia as well as West Africa could not be demonstrated. However, this study has proven that there is a lack of phylogeographic relationship between the haplotype and country of origin. Likewise, previous study has also reported that the Asian and East African *Cx. quinquefasciatus* were assigned to the same genetic cluster, suggesting that heavy human traffic across the Indian Ocean might be the reason for the occurrence of shared genetic lineages (Fonseca *et al*., 2006).

As noted previously, high genetic variability demonstrated in Asian *Cx. quinquefasciatus* (i.e., India, Indonesia and Japan) (Fonseca *et al*., 2006) contrasted the

levels of variability in Malaysian isolates. As inferred from both COI and COII markers, the genetic diversity of Malaysian *Cx. quinquefasciatus* was extremely low since only three haplotypes and four haplotypes were revealed by COI and COII, respectively. Lack of a population genetic structure of this species has also been observed in Bangladesh, where only two haplotypes were revealed by COII marker (Hasan *et al*., 2009). Low levels of genetic diversity might be due to the bottleneck effect that led to the rapid declination of genetic variability (Nei *et al*., 1975). In this context, Malaysian *Cx. quinquefasciatus* might have experienced population bottleneck caused by the vector-borne disease control programs (i.e., larviciding, fogging, indoor residual spray and elimination of breeding sources) conducted in Malaysia since 1967 and consequently reduced the genetic variation. As dengue is the main vector borne disease in Malaysia, specific vector control programs carried out are mainly targeting *Aedes* and not on *Culex* mosquitoes. Therefore, the selection criteria for the study sites in the present study were based on the frequent reports of dengue cases and fogging activities from these sites. The intense fogging activities using broad based insecticides might indirectly suppress the populations of *Cx. quinquefasciatus* as well. Similar findings have been reported by Cartaxo *et al*. (2011), where the lymphatic filariasis vector control programs have reduced the genetic diversity of Brazilian *Cx. quinquefasciatus*, as evidenced by the monitoring of its genetic diversity using microsatellites over a 3-year period. The findings of this previous study suggested physical elimination of breeding sites and larviciding prevent their reproductive success, thus resulting in reduced levels of genetic diversity.

In the context of insecticide resistance, several researchers reported that insecticide resistant-mosquitoes exhibited high degree of genetic variation inferred from random amplified polymorphic DNA (Ocampo & Wesson, 2004; Sharma *et al*., 2009), 16S rRNA sequence (Sharma *et al*., 2010) as well as isozyme loci (Ayres *et al*., 2004).

Although high genetic variation has been observed in these previous studies, it nevertheless does not exclude that fixation of advantageous mutations associated with hitchhiking effect which could take place in other genome regions around a putative insecticide resistance locus (Yan *et al*., 1998). Malaysian *Cx. quinquefasciatus* that were collected concurrently from the same localities have developed a wide range of insecticide resistance towards DDT, propoxur, malathion and permethrin (refer Chapter 5). It is important to point out that insecticide resistance has evolved in this mosquito species in Malaysia, suggesting that the evolution of insecticide resistance is associated with hitchhiking effect and consequently reduced the levels of genetic variation. Hitchhiking effect associated with organophosphate resistance reported previously provide evidence that genetic variation could be rapidly reduced in the populations that have been exposed to insecticides over a relatively long time (Yan *et al*., 1998).

It has also been reported that the bottleneck effect on genetic variation is more accentuated in mitochondrial, in comparison to nuclear loci, as the genetic drift lacks the time to reduce variation at nuclear loci while expansion of population from depletion in number of founder females into a new region (Birungi & Munstermann, 2002). In future, it is important to incorporate additional markers that target nuclear loci to further confirm the population genetic structure of *Cx. quinquefasciatus* in this region.

Apart from insecticide resistance, studies have also proven that the *Wolbachia*-infected *Cx. quinquefasciatus* populations demonstrated a drastic reduction of mitochondrial variation (Rasgon *et al*., 2006; Behbahani, 2012). Given that the infection of *Wolbachia* is commonly found in Asian *Cx. quinquefasciatus* populations (Kittayapong *et al*., 2000; Ravikumar *et al*., 2011), it is suggested that low mitochondrial diversity observed in the present study may be also be contributed by *Wolbachia* infection.

In summary, the present study inferred that haplotype AB1 was the common ancestor and the most widespread haplotype due to its dispersal in Malaysia. Besides, this study has proved that there is a lack of phylogeographic relationship between the haplotype and country of origin. It is suspected that vector control activities have reduced the levels of genetic variability in Malaysian *Cx. quinquefasciatus*. The findings of this study also revealed that COII was more variable and informative, in comparison to COI. Further study with increased number of individuals from wider biogeographic areas in Malaysia and the incorporation of additional markers that are more variable will be beneficial in unraveling the presence of additional haplotypes.

CHAPTER 5

CURRENT SUSCEPTIBILITY STATUS OF MALAYSIAN *CULEX QUINQUEFASCIATUS* AGAINST DDT, PROPOXUR, MALATHION AND PERMETHRIN

5.1 INTRODUCTION

Culex quinquefasciatus is the most common Malaysian nuisance mosquito (Yap *et al*., 2000a). It is also a potential vector of urban lymphatic filariasis caused by the nematode parasite, *Wuchereria bancrofti* in Malaysia (Vythilingam *et al*., 2005). Around the world, its significance as a vector of bancroftian filariasis (Samuel *et al*., 2004), West Nile virus (Sardelis *et al*., 2001; Pitzer *et al*., 2009), Saint Louis encephalitis virus (Jones *et al*., 2002), Ross River virus (Lindsay *et al*., 1993) and Japanese encephalitis virus (Nitatpattana *et al*., 2005) has been well-documented.

Application of organochlorines, organophosphates, carbamates and pyrethroids remains as the main control measure in vector control programs. However, the extensive use of insecticides have contributed to insecticide resistance development through the selection of certain genes (WHO, 2006). In fact, insecticide resistance is not a new phenomenon and is an increasing problem worldwide. *Culex quinquefasciatus* from different parts of the world have been reported to be resistant to various insecticide classes (Bisset *et al*., 1997; Chandre *et al*., 1997, Liu *et al*., 2004; Sathantriphop *et al*., 2006; Kasai *et al*., 2007; Pridgeon *et al*., 2008). Among the various mosquito control approaches, adulticiding with ultra low volume (ULV) fogging, thermal fogging, surface residual spray and household insecticide products are specifically designed for

the control of adult mosquitoes (Yap *et al*., 2000b). In many urban and sub-urban areas, larviciding is the most widely used method for the control of *Cx. quinquefasciatus* larvae, as high levels of adult organochlorine and organophosphate resistance have been reported (Chavasse & Yap, 1997).

To date, no nationwide investigation of insecticide susceptibility status of wild *Cx. quinquefasciatus* has been reported in Malaysia. Over the years, the susceptibility status of wild *Cx. quinquefasciatus* against insecticides has been focused in the Klang Valley (Kuala Lumpur and Selangor), Pahang and Penang districts (Reid, 1955; Wharton, 1958; Thomas, 1962; Lee & Tadano 1994; Lee *et al*., 1997a; Nazni *et al*., 2005). There has been a dearth of information regarding the insecticide susceptibility status of wild *Cx. quinquefasciatus* in other districts of Malaysia. Hence, the present study is the first attempt to quantify the susceptibility status of wild *Cx. quinquefasciatus* against four active ingredients representing four major insecticide classes from each state of Malaysia, including East Malaysia. The findings of this study will be a timely reminder and an early warning to local authorities that systematic insecticide resistance management is essential for the improvement of current vector control operations in Malaysia.

5.2 MATERIALS AND METHODS

5.2.1 MOSQUITO STRAINS

Mosquito larvae were collected from stagnant water at residential areas in each state of Malaysia (Figure 5.1 and Table 5.1), by using a previously described dipping method (Mendoza *et al*., 2008) (refer Chapter 3). Because there is no specific control program for *Culex* spp. mosquitoes in Malaysia, the selection criteria for these study sites were based on the frequent reports of dengue cases and fogging activities from these sites.

Field-collected larvae were transported to the laboratory and reared to adulthood. Larvae were provided with a fine mixture of mice chow, beef liver and milk powder in the ratio of 2:1:1 by weight, while adults were provided with 10% sucrose solution. The emerging *Cx. quinquefasciatus* adults were identified according to illustrated keys (Rattanarithikul *et al*., 2005) and cross-referenced with the voucher specimens from the laboratory. Three days after emergence, the *Cx. quinquefasciatus* female mosquitoes were blood-fed by using a BALB/c mouse. Three days after blood feeding, 300ml capacity oviposition cups containing 200ml deionized water were introduced into mosquito cages (33 x 33 x 33cm). The hatched larvae were designated as first generation (F1), which were subsequently used for larval susceptibility bioassays while adults from F1 reared larvae were used for adult susceptibility bioassays. For comparison purposes, a laboratory reference strain of *Cx. quinquefasciatus* from the Institute for Medical Research, Kuala Lumpur, which has been cultured under insecticide-free conditions for 117 generations was used.

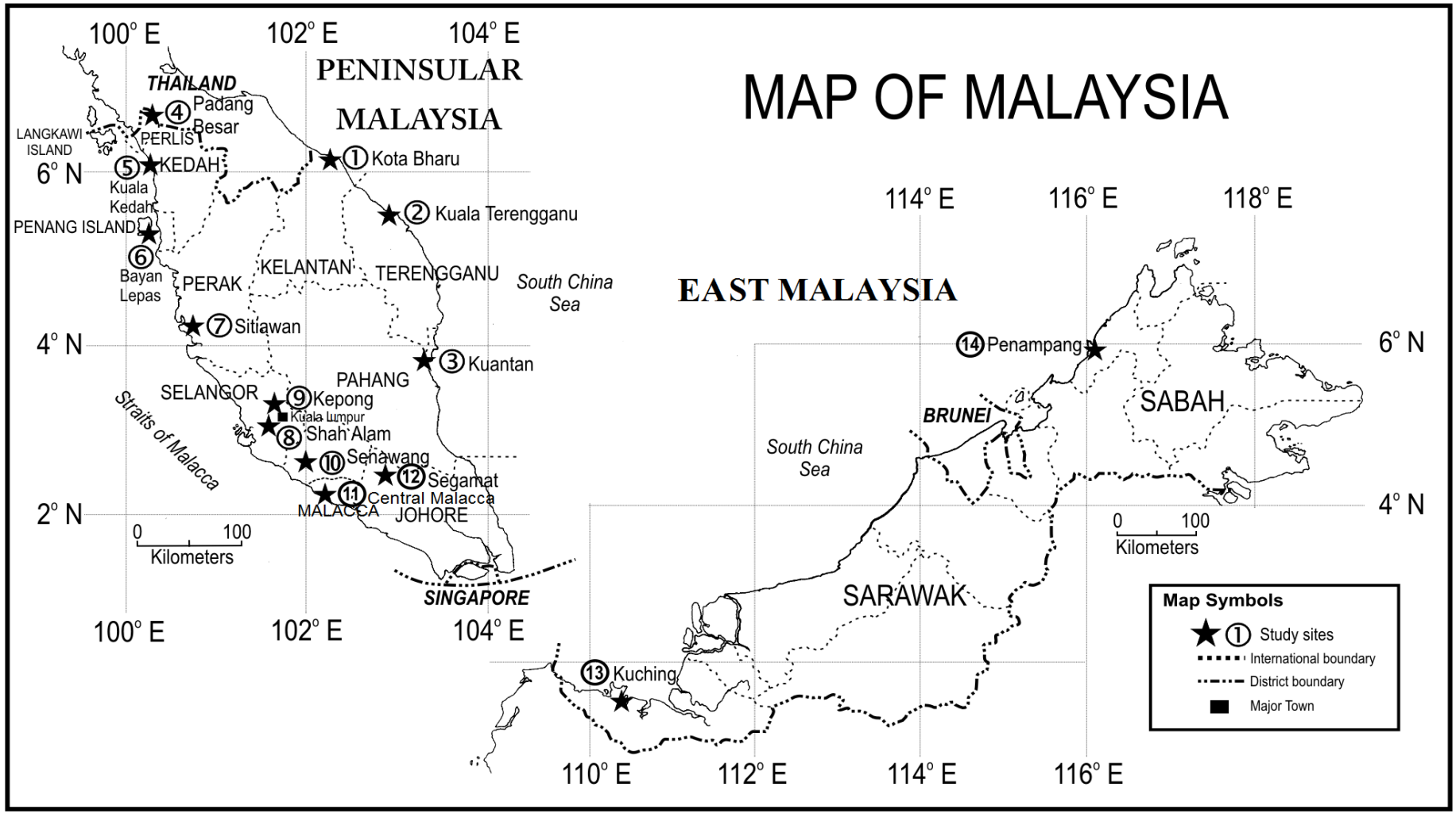

Figure 5.1 Collection sites of *Cx. quinquefasciatus* larvae in Malaysia.

Table 5.1 Geographical description of mosquito collection sites across 14 states in Malaysia.

Malaysia	Region	State	District	Study Site	GPS Coordinates	Landscape
Peninsular	East Coast	Kelantan	Kota Bharu	Taman Guru	06 °05’49.43”N, 102°14’06.80”E	Sub-urban
		Terengganu	Kuala Terengganu	Kg. Simpang Empat	05°15’57.73”N, 103°10’49.90”E	Rural
		Pahang	Kuantan	Taman Chenderawasih	03°48’00.40”N, 103°18’02.20”E	Sub-urban
	Northern	Perlis	Padang Besar	Taman Singgahsana	06°39’11.00”N, 100°18’54.00”E	Rural
		Kedah	Kuala Kedah	Taman Selat	06°05’02.10”N, 100°18’07.70”E	Sub-urban
		Penang	Bayan Lepas	Taman Bayan Baru	05°19’46.51”N, 100°17’24.80”E	Urban
		Perak	Sitiawan	Taman Bunga Ros	04°12’42.21”N, 100°41’42.20”E	Sub-urban
	Central	Selangor	Shah Alam	Section 17	03°02’58.28”N, 101°30’16.40”E	Urban
		Kuala Lumpur	Kepong	Kepong Baru	03°12’18.23”N, 101°38’43.60”E	Urban
	Southern	Negeri Sembilan	Senawang	Taman Marida	02°41’52.40”N, 101°59’02.44”E	Sub-urban
		Malacca	Central Malacca	Kg. Pengkalan Rama Pantai	02°12’35.77”N, 102°15’02.52”E	Rural
		Johore	Segamat	Segamat Baru	02°29’56.50”N, 102°51’12.10”E	Sub-urban
East Malaysia	West	Sarawak	Kuching	Taman Budaya	01°33’10.11”N, 110°20’41.00”E	Sub-urban
	East	Sabah	Penambang	Bundusan Villa	05°56’22.34”N, 116°06’19.37”E	Sub-urban

5.2.2 INSECTICIDES

Four active ingredients representing four major insecticide classes used in both larval and adult susceptibility tests. These included an organochlorine (DDT), a carbamate (propoxur), an organophosphate (malathion) and a pyrethroid (permethrin). DDT 4.0%, propoxur 16%, malathion 8% and permethrin 0.5% in solution form and the Whatman No.1 filter papers (12 x 15cm) that were impregnated with 2ml of the diagnostic concentrations of DDT 4.0%, propoxur 0.1%, malathion 5.0% and permethrin 0.25%, respectively, were purchased from WHOPES Collaborating Centre in Universiti Sains Malaysia, Penang.

5.2.3 LARVAL SUSCEPTIBILITY TEST

This test was conducted according to the WHO (1981a) larval susceptibility bioassay procedure. Stock solutions of each insecticide were made up in ethanol and further diluted with the desired concentrations. Briefly, the bioassay was conducted in 300ml disposable paper cups. The prepared stock solution of insecticide was added into 150ml deionized water. Five concentrations and 3 containers (25 late third or early fourth instar larvae per replicate) per concentration were performed with each insecticide, i.e., DDT (0.500-4.400mg/l); propoxur (0.030-1.400mg/l); malathion (0.020-1.900mg/l); permethrin (0.030-1.800mg/l). After introducing the larvae into paper cups, water was added to make the final volume to 250ml. The control (untreated) was set up by adding 1ml of ethanol into the paper cups containing 249ml deionized water. Larval mortality was recorded after 24h of continuous exposure. Moribund larvae were counted as dead.

5.2.4 ADULT SUSCEPTIBILITY TEST

This test was conducted according to the WHO (1981b) adult susceptibility bioassay procedure, with minor modifications. A batch of 15 sucrose-fed, 3- to 5-day-old female mosquitoes was exposed to the diagnostic WHO-impregnated papers and the test was repeated three times. Briefly, the mosquitoes were removed from the cage by using a plastic aspirator tube and transferred into WHO exposure tubes (125mm in length, 44mm in diameter). Test tubes were covered with black cloth to ensure that mosquitoes would rest on the impregnated paper. For the determination of KT_{50} (50% knockdown time) value, the number of mosquitoes knocked down was recorded every minute (Lee *et al*., 1997a, Nazni *et al*., 2009) for DDT, propoxur, malathion and permethrin during exposure periods of 4 hours, 2 hours, 1 hour and 3 hours, respectively. Mosquitoes that survived the cumulative exposure period were transferred to WHO holding tubes to allow an observation of post-treatment effect. Controls were exposed to non-treated paper. Cotton pads soaked in 10% sugar solution were provided during the 24h post-exposure period. Mortality was recorded 24h after the initial exposure period.

5.2.5 STATISTICAL ANALYSIS

Larval bioassay data within the range of 5-95% were subjected to probit analysis (Finney, 1971) using a computerized program, PROBIT (National Center for Scientific Research, France) developed by Raymond (1993). Based on the LC_{50} obtained from larval bioassays, resistance ratios (RR) were calculated by dividing values for the resistant strain by those of the susceptible strain (Brown & Pal, 1971). Calculated RR values > 10 are indicative of high resistance, 5-10 are indicative of medium resistance and < 5 are indicative of low resistance (Mazarri & Georghiou, 1995). The associations

between the RR values in larval bioassays were accessed by Spearman rank-order correlation, for the determination of cross-resistance, as described by Bisset *et al*. (1997).

With regard to adult bioassays, a specific time for each chemical's knockdown analysis was performed based on the KD of the reference strain. To best describe the susceptibility status, knockdown evaluation of DDT, propoxur, malathion and permethrin were performed at 80, 50, 70 and 50% of the total exposure time, respectively. The percentage mortality at 24 hours post-treatment was used to determine susceptibility status: 98-100% mortality indicates susceptibility, 80-97% mortality suggests the possibility of resistance that needs to be further confirmed and less than 80% mortality suggests resistance (WHO, 2009c). Abbott's formula (Abbott, 1925) was applied to correct percentage mortality if control mortality was more than 5%. Comparative measure of knockdown and mortality between the study sites was performed by one-way ANOVA (dependent variable = knockdown/mortality; factor = study site). Tukey's test was used to separate means in significant ANOVAs, $P < 0.05$. Spearman rank-order correlation between the mortality percentages in adult bioassays were performed for the determination of cross-resistance (Bisset *et al*., 1997).

5.3 RESULTS

The susceptibility status of *Cx. quinquefasciatus* against DDT, propoxur, malathion and permethrin in larval and adult stages are presented in Table 5.2 and Table 5.3, respectively. In each insecticide tested, both larval and adult bioassays exhibited dissimilar trends in susceptibility across all study sites. Various insecticide susceptibility levels (susceptible, low to high resistance) in both larval and adult bioassays were demonstrated from different localities in Malaysia.

Larval bioassays demonstrated various resistance ratios, ranging from 0.66 to 3.83, 0.38 to 2.93, 0.36 to 13.88 and 0.23 to 3.81 fold for DDT, propoxur, malathion and permethrin, respectively. It is important to point out that the *Cx. quinquefasciatus* larvae from Terengganu were susceptible to all four insecticides, having resistance ratios < 1. The *Cx. quinquefasciatus* larvae from Kuala Lumpur, Selangor, Malacca, Penang and Negeri Sembilan were most resistant to malathion by exhibiting resistance ratios > 10. In addition, Spearman rank-order correlation indicated a significant correlation between resistance ratios of propoxur and malathion (r = 0.780, P = 0.001) and between resistance ratios of propoxur and permethrin (r = 0.613, P = 0.020) in larval bioassays (Figure 5.2), while no correlation was found with other insecticides in either larval or adult bioassays.

In adult bioassays, DDT resistance was expressed most frequently among the four insecticides evaluated, as 0% knockdown was recorded at 80% of the total exposure time from 12 out of 14 of the populations. Meanwhile, 0% knockdown was detected from 8 out of 14 and 5 out of 14 of the populations using propoxur at 50% of the total exposure time and malathion at 70% of the total exposure time, respectively. A wide spectrum of knockdown was detected with permethrin evaluated at 50% of the total exposure time across all study sites.

Across all study sites, DDT and propoxur exhibited less than 40% and 70% mortality, respectively, whereas complete mortality was observed in malathion and permethrin from a few populations (Table 5.3). The results indicated that *Cx. quinquefasciatus* was most susceptible to permethrin. One-way ANOVA revealed that the susceptibility status of *Cx. quinquefasciatus* adults to various insecticides were significantly different across all study sites.

With respect to both larval and adult bioassays that showed similar trends in susceptibility, both *Cx. quinquefasciatus* larvae and adults from Kelantan, Terengganu and Perlis were susceptible to malathion. *Culex quinquefasciatus* from Kuala Lumpur, Selangor, Malacca, Penang and Negeri Sembilan were also apparently resistant to malathion with resistance ratios > 10 in larval bioassays and low knockdown rate observed in adult bioassays. Meanwhile, inconsistency of mosquito susceptibility in both larval and adult stages from other districts against other insecticides was recorded.

Table 5.2 DDT, propoxur, malathion and permethrin susceptibility for several Malaysian *Cx. quinquefasciatus* larval strains.

Strain	DDT		Propoxur		Malathion		Permethrin	
	LC_{50} (mg/l) 95% (C.L.)	RR_{50}	LC_{50} (mg/l) 95% (C.L.)	RR_{50}	LC_{50} (mg/l) 95% (C.L.)	RR_{50}	LC_{50} (mg/l) 95% (C.L.)	RR_{50}
Reference	1.009 (1.057-1.143)	–	0.242 (0.234-0.251)	–	0.124 (0.114-0.135)	–	0.211 (0.167-0.196)	–
Kelantan	1.226 (1.134-1.137)	1.12*	0.416 (0.382-0.454)	1.72*	0.045 (0.041-0.049)	0.36**	0.714 (0.564-0.895)	3.38*
Terengganu	0.968 (0.846-1.122)	0.88	0.092 (0.069-0.115)	0.38**	0.057 (0.046-0.070)	0.46**	0.143 (0.119-0.169)	0.68
Pahang	2.758 (2.709-2.802)	2.51*	0.168 (0.146-0.189)	0.69**	0.168 (0.146-0.189)	1.35*	0.177 (0.152-0.203)	0.84
Perlis	3.440 (3.355-3.535)	3.13*	0.146 (0.116-0.177)	0.60**	0.055 (0.051-0.060)	0.44**	0.049 (0.045-0.055)	0.23**
Kedah	3.508 (3.418-3.589)	3.19*	0.489 (0.442-0.542)	2.02*	0.864 (0.812-0.913)	6.97*	0.187 (0.162-0.216)	0.89
Penang	1.325 (1.230-1.417)	1.21*	0.708 (0.609-0.809)	2.93*	1.271 (1.216-1.322)	10.25*	0.375 (0.326-0.429)	1.78*
Perak	0.725 (0.618-0.898)	0.66**	0.175 (0.153-0.196)	0.72**	0.541 (0.514-0.568)	4.36*	0.056 (0.051-0.063)	0.27**
Selangor	3.155 (3.043-3.284)	2.87*	0.656 (0.580-0.725)	2.71*	1.650 (1.592-1.714)	13.31*	0.803 (0.706-0.910)	3.81*
Kuala Lumpur	3.067 (2.991-3.175)	2.79*	0.623 (0.569-0.687)	2.57*	1.721 (1.651-1.806)	13.88*	0.472 (0.426-0.518)	2.24*
Negeri Sembilan	4.205 (4.160-4.251)	3.83*	0.456 (0.390-0.571)	1.88*	1.247 (1.203-1.300)	10.06*	0.504 (0.457-0.558)	2.39*
Malacca	3.695 (3.656-3.735)	3.36*	0.463 (0.413-0.514)	1.91*	1.309 (1.240-1.372)	10.57*	0.430 (0.401-0.463)	2.04*
Johore	3.705 (3.664-3.747)	3.37*	0.607 (0.547-0.675)	2.51*	1.009 (0.964-1.059)	8.14*	0.389 (0.337-0.451)	1.84*
Sarawak	2.684 (2.587-2.781)	2.44*	0.471 (0.426-0.517)	1.95*	0.351 (0.306-0.396)	2.83*	0.428 (0.406-0.449)	2.03*
Sabah	1.064 (0.974-1.159)	0.97	0.384 (0.332-0.446)	1.59*	0.700 (0.647-0.759)	5.65*	0.137 (0.108-0.174)	0.65

C.L. = Confidence Limit. Reference strain was obtained from Institute for Medical Research, Kuala Lumpur, Malaysia. Mosquito larvae collected from the field were reared to F1. Asterisk * indicates C.L. does not overlap with the reference strain and significantly different from the reference strain. Asterisk ** indicates those strains with a significant lower resistance ratio.

Table 5.3 Knockdown and mortality of larval-reared *Cx. quinquefasciatus* adults using a WHOPES treated filter paper assay.

	Knockdown (%)				Mortality (%)			
Strain	**DDT 4.0%**	**Propoxur 0.1%**	**Malathion 5.0%**	**Permethrin 0.25%**	**DDT 4.0%**	**Propoxur 0.1%**	**Malathion 5.0%**	**Permethrin 0.25%**
Reference	4.44 ±4.44	75.55 ±9.69	51.11 ±2.22	86.67 ±3.85	43.34 ±2.72	100.00 ±0.00	100 ±0.00	100.00 ±0.00
Kelantan	0.00 ±0.00^{a}	0.00 ±0.00^{a}	6.67 ±0.00^{ab}	20.00 ±6.67^{ab}	^{R}24.44 ±4.44^{cde}	^{R}3.34 ±3.34^{a}	^{M}96.67 ±3.34^{ef}	^{R}43.33 ±10.00^{ab}
Terengganu	0.00 ±0.00^{a}	50.00 ±10.00^{c}	40.00 ±0.00^{e}	35.00 ±5.00^{abc}	^{R}25.00 ±5.00^{cde}	^{R}55.00 ±5.00^{cd}	^{S}100.00 ±0.00^{f}	^{M}95.00 ±5.00^{de}
Pahang	0.00 ±0.00^{a}	0.00 ±0.00^{a}	33.33 ±3.33^{de}	26.67 ±10.19^{abcd}	^{R}13.33 ±3.33^{abc}	^{R}20.00 ±5.77^{ab}	^{S}100.00 ±0.00^{f}	^{R}36.67 ±3.33^{a}
Perlis	0.00 ±0.00^{a}	22.22 ±4.45^{bc}	26.67 ±3.85^{cde}	24.45 ±2.22^{ab}	^{R}2.22 ±2.22^{ab}	^{R}68.89 ±5.88^{d}	^{R}75.55 ±2.22^{def}	^{R}71.11 ±2.22^{cd}
Kedah	0.00 ±0.00^{a}	0.00 ±0.00^{a}	13.33 ±3.85^{abc}	22.22 ±5.88^{ab}	^{R}4.45 ±2.22^{ab}	^{R}6.67 ±3.85^{a}	^{R}71.11 ±5.88^{de}	^{R}37.78 ±2.22^{ab}
Penang	0.00 ±0.00^{a}	0.00 ±0.00^{a}	0.00 ±0.00^{a}	20.00 ±0.00^{abc}	^{R}35.56 ±5.88^{de}	^{R}37.78 ±2.22^{bc}	^{R}35.56 ±5.88^{bc}	^{M}76.67 ±10.00^{cde}
Perak	0.00 ±0.00^{a}	0.00 ±0.00^{a}	0.00 ±0.00^{a}	36.67 ±3.34^{abcd}	^{R}20.00 ±0.00^{bcd}	^{R}6.67 ±6.67^{a}	^{R}10.00 ±3.33^{ab}	^{M}83.33 ±10.00^{cde}
Selangor	0.00 ±0.00^{a}	8.89 ±2.22^{ab}	0.00 ±0.00^{a}	35.56 ±4.44^{abd}	^{R}6.67 ±3.85^{abc}	^{R}55.55 ±2.22^{cd}	^{R}0.00 ±0.00^{a}	^{R}62.22 ±4.45^{bc}
Kuala Lumpur	0.00 ±0.00^{a}	4.45 ±2.22^{a}	0.00 ±0.00^{a}	13.33 ±3.85^{a}	^{R}17.78 ±2.22^{abcd}	^{R}33.33 ±3.85^{bc}	^{R}11.11 ±5.88^{ab}	^{R}77.78 ±2.22^{cde}
Negeri Sembilan	2.22 ±2.22^{a}	0.00 ±0.00^{a}	0.00 ±0.00^{a}	5.00 ±5.00^{ab}	^{R}20.00 ±0.00^{bcd}	^{R}10.00 ±5.77^{a}	^{R}6.67 ±6.67^{ab}	^{S}100.00 ±0.00^{e}
Malacca	0.00 ±0.00^{a}	0.00 ±0.00^{a}	2.33 ±2.22^{a}	35.55 ±9.69^{abcd}	^{R}11.11 ±2.22^{abc}	^{R}15.55 ±2.22^{ab}	^{R}4.44 ±4.44^{ab}	^{M}82.22 ±5.88^{cde}
Johore	0.00 ±0.00^{a}	0.00 ±0.00^{a}	2.22 ±2.22^{a}	70.00 ±3.33^{cd}	^{R}2.22 ±2.22^{ab}	^{R}22.22 ±2.22^{abc}	^{R}31.11 ±4.44^{bc}	^{S}100.00 ±0.00^{e}
Sarawak	0.00 ±0.00^{a}	13.33 ±3.85^{ab}	20.00 ±6.67^{cde}	75.56 ±5.88^{d}	^{R}0.00 ±0.00^{a}	^{R}53.33 ±3.85^{d}	^{R}62.22 ±5.88^{d}	^{S}100.00 ±0.00^{e}
Sabah	28.89 ±2.22^{b}	35.56 ±4.44^{c}	24.44 ±4.44^{bcd}	56.67 ±3.34^{bcd}	^{R}40.00 ±3.85^{e}	^{R}62.22 ±4.45^{d}	^{R}55.55 ±2.22^{cd}	^{M}93.33 ±0.00 ^{d}
One way ANOVA	F = 74.53 df = 13, 28 P < 0.0001	F = 23.13 df = 13, 28 P < 0.0001	F = 18.07 df = 13, 28 P < 0.0001	F = 1026.00 df = 13, 28 P < 0.0001	F = 13.40 df = 13, 28 P < 0.0001	F = 28.44 df = 13, 28 P < 0.0001	F = 48.16 df = 13, 28 P < 0.0001	F = 24.60 df = 13, 28 P < 0.0001

Means followed by a different letter were significantly different, $P < 0.05$, Tukey's test. R = resistant, S = susceptible, M = moderate resistant as determined by WHO (2009c).

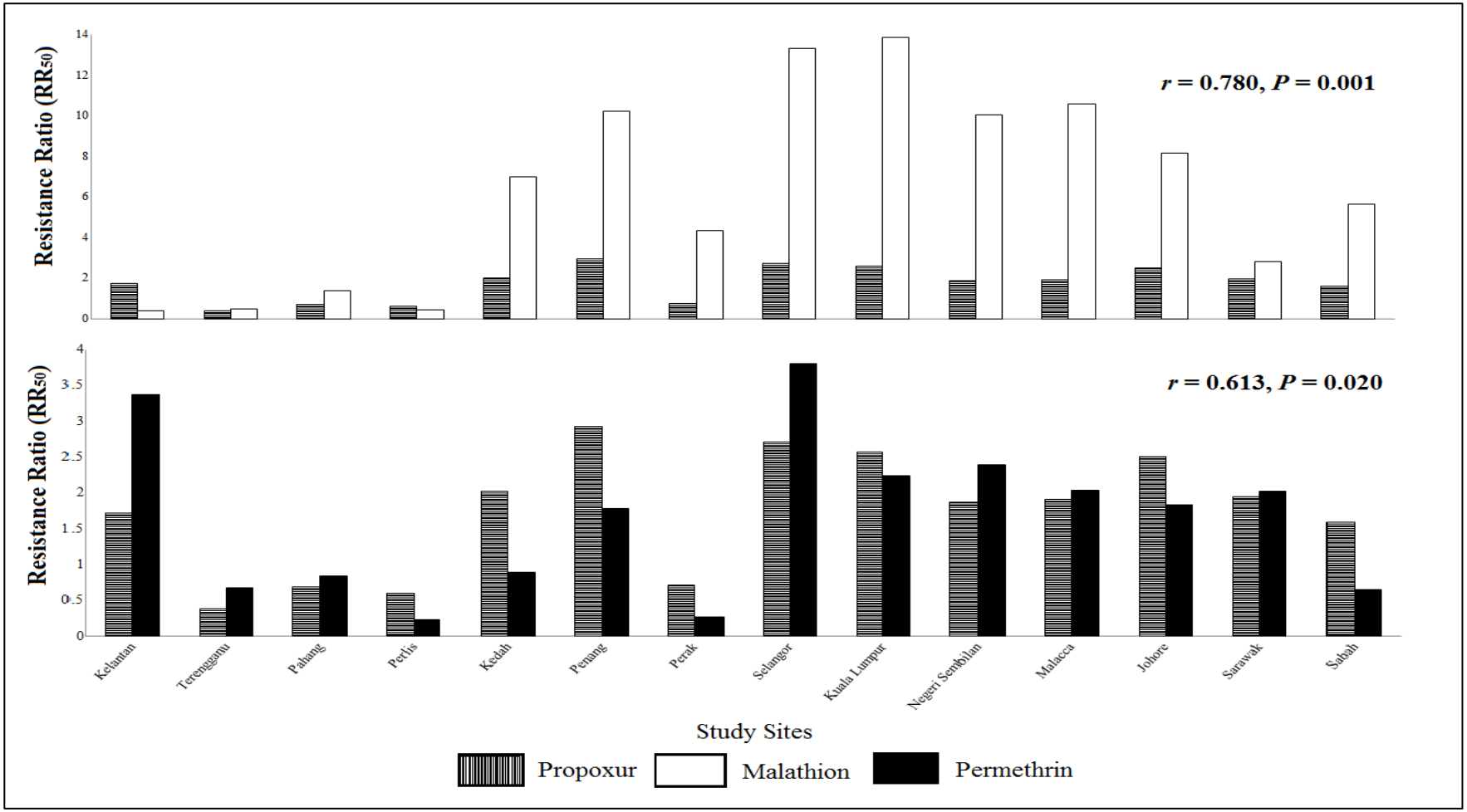

Figure 5.2 Spearman rank-order correlation between resistance ratio of *Cx. quinquefasciatus* larvae against propoxur, malathion and permethrin.

5.4 DISCUSSION

In the present study, mosquitoes from collection sites across Malaysia evaluated in both larval and adult bioassays exhibited dissimilar trends in susceptibility against the four insecticides tested. The occurrence of these incidences might be due to the differences between the insecticide resistance gene expression in larval and adult stages. A number of studies have indicated that insecticide resistance is more accentuated in the larval stage (Nazni *et al*., 2005; Selvi *et al*., 2006; 2007; Li & Liu 2010) while a lack of expression was observed in the adult stage (Huchard *et al*., 2006). However, higher levels of insecticide resistance in the adult stage also have been observed (Chavasse & Yap, 1997). Cross-stage resistance has been reported due to the overlapping of certain mechanisms in response to insecticide pressure (Li & Liu, 2010).

Generally, among the four insecticides tested in this study, Malaysian *Cx. quinquefasciatus* larvae were most resistant to malathion. Several conventional organophosphates have been introduced as larvicides for the control of mosquito larvae in Malaysia (Yap *et al*., 2000b). The occurrence of high level malathion resistance in the larval stage may be due to the over-usage of organophosphorus insecticides, resulting in the selection of one or more genes within exposed mosquitoes due to the use of compounds that share the same mode of action (Liu *et al*., 2004, Selvi *et al*., 2005). In the current study, malathion resistance was more accentuated in larval stage, as higher levels of malathion resistance was demonstrated in larvae, compared to adults. Likewise, a previous study also reported higher levels of malathion resistance in the larval stage (Selvi *et al*., 2005). However, manifold expression of organophosphate resistance in *Cx. quinquefasciatus* adults has been frequently reported (Chavasse & Yap, 1997). Moreover, the increasing trend of esterase activities from the egg to adult stage has been observed in malathion resistant strains (Selvi *et al*., 2007). Hence, biochemical

test should be conducted to identify the malathion resistance mechanism in Malaysian *Cx. quinquefasciatus* populations. Statistical analysis indicated that there was a significant correlation between propoxur and permethrin resistance and between propoxur and malathion resistance, suggesting the presence of cross-resistance. Although cross-resistance between propoxur and permethrin in this species has been observed previously (Sathantriphop *et al*., 2006), the actual mechanism(s) that caused this phenomenon remain questionable. However, cross-resistance between pyrethroid and carbamate in *Anopheles funestus* Giles has been reported and suggested that elevated levels of mixed function oxidases conferred cross-resistance in both classes of insecticides (Brooke *et al*., 2001, Cuamba *et al*., 2010). Conversely, cross-resistance between organophosphates and carbamates in *Cx. quinquefasciatus* has been documented frequently (Bisset *et al*., 1990; Chandre *et al*., 1997; Liu *et al*., 2004; Selvi *et al*., 2005). In addition to identifying the resistance gene that conferred organophosphate and carbamate resistance, acetylcholinesterase (AChE) insensitivity has been confirmed through molecular characterization (Cui *et al*., 2006; Alout *et al*., 2007).

Adult bioassays indicated that Malaysian *Cx. quinquefasciatus* were highly resistant to DDT. The laboratory reference strain also exhibited low susceptibility to DDT (% mortality = 43.34). Several mosquito species have expressed and maintained DDT resistance. Nazni *et al*. (2005) documented high DDT KT_{50} values in a laboratory reference strain and suggested that DDT was the least effective insecticide among all tested insecticides. Although DDT applications as indoor residual spraying was stopped in Malaysia in 1998, the resistance phenotype still remains in this mosquito population, suggesting that DDT would be ineffective. Similarly, a DDT resistance phenotype remained in a laboratory reference strain of *Aedes aegypti* Linnaeus although this strain has been cultured under insecticide-free conditions for 1,014 generations (Nazni *et al*.,

2009). Furthermore, high levels of DDT resistance persist in *Cx. pipiens* populations from Egypt, although this insecticide has not been used since the 1970s (Zayed *et al*., 2006).

Among the four insecticides tested in this study, *Cx quinquefasciatus* was most susceptible to permethrin. However, low permethrin resistance was detected in these populations. Pyrethroids are the most important class of insecticide with major usage in public health and household insecticide products (Yap *et al*., 2000b). The extensive usage of this insecticide may lead to pyrethroid resistance development in this species. In 1996, the introduction of permethrin fogging activities contributed to permethrin resistance development in *Cx. quinquefasciatus* (Nazni *et al*., 1998). Moreover, as this species prefers to rest indoors (Tham, 2000), it is more likely to be exposed to pyrethroid-based household insecticide products. This is further supported by Yap *et al*. (1995), where *Cx. quinquefasciatus* was most tolerant to household insecticide products containing pyrethroids as the active ingredient. Several formulations of household insecticide products such as coils, mats, liquid vaporizer and aerosol have been introduced widely in Malaysian markets. The mentioned formulations contained the active ingredient of d-allethrin, d-trans allethrin, transfluthrin, prallethrin, s-bioallethrin, deltamethrin, d-phenothrin, permethrin and tetramethrin (Yap *et al*., 2000b). It is suggested that the over-reliance of these pyrethroid-based household insecticide products conferred the low permethrin resistance detected in this study.

The current findings indicated that *Cx. quinquefasciatus* from Selangor and Kuala Lumpur exhibited a similar trend of resistance against malathion. In recent years, a similar study showed that *Cx. quinquefasciatus* larvae and adults from Kuala Lumpur were highly resistant to malathion (Nazni *et al*., 2005). To date, numerous dengue and chikungunya cases from the areas of Kuala Lumpur and Selangor have been frequently reported to the Ministry of Health, Malaysia. In order to control the spread of these

mosquito-borne pathogens, fogging activities have been frequently carried out in these endemic areas. As a consequence, *Cx. quinquefasciatus* may have developed insecticide resistance through this selection pressure. Due to the high frequency of fogging activities, these areas also were targeted for insecticide resistance studies by Chen *et al*. (2005b; 2005c) and Nazni *et al*. (2005), which provides a strong comparison to the current study.

Several resistance reports of Malaysian wild *Cx. quinquefasciatus* against DDT (Reid, 1955; Thomas, 1962; Nazni *et al*., 2005), propoxur (Nazni *et al*., 2005), malathion (Lee *et al*., 1997a; Nazni *et al*., 2005) and permethrin (Lee *et al*., 1997a; Nazni *et al*., 2005) have been reported previously in a few states in Malaysia, although these previous results could not be compared directly due to different handling methods and procedures. In the current study, insecticide susceptibility status of Malaysian *Cx. quinquefasciatus* larvae and adults has been demonstrated throughout the country, indicating that different localities should be targeted with different chemicals. The findings of the current study may assist local authorities by providing an updated susceptibility baseline and data to be used for choosing application rates and insecticides for vector control operations.

Although the resistance ratio reported from most of the study sites could be considered low, it nevertheless indicates that resistance is developing and preventive measures should be considered proactively. However, insecticide resistance was detected in several populations, thereby allowing for biochemical and molecular studies to characterize the mechanism involved in *Cx. quinquefasciatus* resistance.

CHAPTER 6

BIOCHEMICAL CHARACTERIZATION OF INSECTICIDE RESISTANCE MECHANISMS IN MALAYSIAN *CULEX QUINQUEFASCIATUS*

6.1 INTRODUCTION

Insecticide resistance mechanisms have been the subject of research interest among researchers from different parts of the world, including Malaysia. It has been proven that increased levels of mixed function oxidases contribute resistance to four major insecticide classes (i.e., organochlorines, carbamates, organophosphates and pyrethroids) (Brewer & Keil, 1989; Brooke *et al*., 2001; Fonseca-Gonz âlez *et al*., 2009). Besides, it has been reported that elevated levels of esterases were responsible for the resistance to organophosphates, carbamates and pyrethroids (Peiris & Hemingway, 1993; Achaleke *et al*., 2009). Involvement of glutathione-S-transferase in resistance to organophosphates, organochlorines and pyrethroids has also been noted (Hemingway *et al*., 1991; Zayed *et al*., 2006; Che-Mendoza *et al*., 2009). On the other hand, previous studies have provided evidence on the role of insensitive acetylcholinesterase in resistance to organophosphates and carbamates (Bourguet *et al*., 1996; Pethuan *et al*., 2007). In Malaysia, a considerable amount of research indicated that Malaysian mosquitoes have demonstrated variable biochemical mechanisms in resistance to various insecticide classes (Lee, 1990; Lee *et al*., 1992; 1996; Lee & Tadano, 1994; Lee & Chong, 1995; Nazni *et al*., 1998; 2000; 2004; Selvi *et al*., 2007; Chen *et al*., 2008; Wan-Norafikah *et al*., 2008; 2010).

With regard to *Cx. quinquefasciatus*, it has been incriminated as one of the three 'world's resistant mosquitoes' (APRD, 2013). The first documented case of insecticide resistance (towards organochlorines) in this mosquito species was reported in 1952 in California (Gjullen & Peter, 1952). Subsequently, the widespread development of its biotypes with resistance to 35 insecticide active ingredients were documented worldwide (APRD, 2013). In particular, Malaysian *Cx. quinquefasciatus*, the most abundant and annoying mosquito (Yap *et al*., 2000a) has developed resistance towards four major insecticide classes (Reid 1955; Wharton 1958; Thomas 1962; Lee *et al*., 1997a; Nazni *et al*., 2005).

Enzyme microassay has been commonly used due to its rapid, simple and sensitive method for the identification of mechanisms underlying the insecticide resistance in mosquito population even at low frequencies (Brogdon, 1989; Lee, 1990). However, in Malaysia, the characterization of biochemical mechanisms of *Cx. quinquefasciatus* has been restricted to the district of Kuala Lumpur (Lee, 1990; Lee *et al*., 1992; 1996; Lee & Tadano, 1994; Nazni *et al*., 1998), Sarawak (Nazni *et al*., 2004) and laboratory insecticide selected strains (Lee & Chong, 1995; Nazni *et al*., 1998; Selvi *et al*., 2007). Indeed, there has been a lack of evidence regarding the underlying mechanisms that are involved in insecticide resistance in the field populations of *Cx. quinquefasciatus* from other districts in Malaysia. Although previous studies have identified the roles of certain enzymes in insecticide resistance development, there have been no comprehensive studies which concurrently investigate the roles of α-esterases, β-esterases, mixed function oxidases, glutathione-S-transferase and insensitive acetylcholinesterase in resistance to four major insecticide classes. It is of great concern that the biochemical mechanisms in Malaysian *Cx. quinquefasciatus* populations could be underestimated, especially when there is an occurrence of multiple resistance mechanisms within the same population.

Multiple resistance to a broad spectrum of insecticides (i.e., DDT, propoxur, malathion and permethrin) ranging from susceptible, low to high resistance has been detected in Malaysian *Cx. quinquefasciatus* (refer Chapter 5). However, the actual mechanism(s) that conferred the development of insecticide resistance in these populations remain questionable. In this context, a nationwide investigation was further conducted to (1) quantify the enzyme activities in field populations of *Cx. quinquefasciatus*, as part of an ongoing insecticide resistance monitoring from 14 residential areas across 11 states and one federal territory in Peninsular Malaysia and two states in East Malaysia and thereby attempting to (2) correlate the degree of insecticide resistance with the levels of enzyme activities in this mosquito species. The present study is the first attempt to investigate the roles of α-esterases, β-esterases, mixed function oxidases, glutathione-S-transferase and insensitive acetylcholinesterase towards resistance to DDT, propoxur, malathion and permethrin in *Cx. quinquefasciatus* from all states in Malaysia. Identification of mechanisms underlying the insecticide resistance will be beneficial in developing effective mosquito control programs in Malaysia.

6.2 MATERIALS AND METHODS

6.2.1 MOSQUITO STRAINS

In the present study, a total of 1,440 adult *Cx. quinquefasciatus* with 24 individual mosquitoes representing each of the 60 strains (four enzyme microassays for each population, including laboratory reference strain) were used.

6.2.2 ENZYME MICROASSAYS

Non-specific esterases enzyme microassay was carried out according to established protocols (Brogdon *et al*., 1988; Lee, 1990). A total of 24 individual mosquitoes were homogenized in phosphate buffer solution and were centrifuged at 15,000 rpm for 10 minutes at 4℃. Four aliquots of homogenate (50µl) from each individual mosquito were obtained in this assay. The 50µl of substrate solution (either α-naphthyl acetate or β-naphthyl acetate) was placed in a 96 well plate and left to stand for one minute, followed by the addition of 50µl of 3mM indicator solution (fast blue B salt). The reaction was further incubated for 10 minutes and was stopped by the addition of 50µl of 10% acetic acid. The optical density was measured at 450nm using absorbance microplate reader (BIO-TEK® ELx800™).

Mixed function oxidases enzyme microassay was performed according to the method described by Brogdon *et al*. (1997). A total of 24 individual mosquitoes were homogenized in sodium acetate buffer solution and four aliquots of homogenate (100µl) from each individual mosquito were obtained in this assay. The optical density was measured at 630nm after five minutes incubation of individual mosquito homogenate in each well with 200µl of 2mM 3,3'5,5'-tetramethylbenzidine (TMBZ) and 25µl of 3% hydrogen peroxide.

Glutathion-S-transferase enzyme microassay was conducted according to previously described protocol (Lee & Chong, 1995). A total of 24 individual mosquitoes were homogenized in potassium phosphate buffer solution and were centrifuged at 14,000 rpm for 10 minutes at 4℃. Four aliquots of homogenate (100µl) from each individual mosquito were placed in a 96 well plate, followed by the addition of 50µl of 2mM glutathione and 50µl of 1mM 1-chloro-2, 4-dinitrobenzene (CDNB).

The reaction was further incubated for 30 minutes, followed by the measurement of optical density at 400nm.

With regard to insensitive acetylcholinesterase, enzyme microassay was performed according to the method of Brogdon *et al*. (1988), with minor modifications. Briefly, first batch of 12 individuals mosquitoes were homogenized in potassium phosphate buffer and were centrifuged at 14,000 rpm for 10 minutes at 4℃. In this assay, a total of eight aliquots of homogenate (50µl) from each individual mosquito were obtained. A 50µl of reaction mixture containing 10% acetone buffer solution of 2.6mM acetylthiocholine iodide (ACTHI), 0.3mM of 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) and 0.1% propoxur inhibitor were added into each well. As for positive control, a 50µl of reaction mixture without inhibitor was designed. The reaction was incubated at room temperature (28℃) for 30 minutes, followed by the measurement of optical density at 400nm. This procedure was repeated for the second batch of 12 individuals mosquitoes.

6.2.3 STATISTICAL ANALYSIS

Spearman rank-order correlation was performed to (1) determine the associations between the survivability rates in adult bioassays and enzyme activities, (2) investigate the relationships between enzyme activities.

Based on the mean enzyme level, resistance ratios (RR) were calculated by dividing values for the field strain by those of the laboratory reference strain. Calculated RR values > 1 are indicative of resistance, while values ≤ 1 are indicative of susceptible (Chen *et al*., 2008). Comparative measure of mean enzyme activities between the study sites was performed by one-way analysis of variance (ANOVA) using SPSS version 18. Tukey's test was used to separate means in significant ANOVAs, $P < 0.05$.

Independent-samples t-test was performed to indicate significant increase in mean differences.

With respect to insensitive acetylcholinesterase analysis, individual mosquitoes with more than 70% remaining activity after propoxur inhibition are indicative of homozygous resistance (RR), 30-70% remaining activity are indicative of heterozygous (RS) and less than 30% remaining activity are indicative of homozygous susceptible (SS). Because of the light absorbance of propoxur in the microplate, in certain cases, homogenates appear to show higher acetylcholinesterase activity in propoxur-inhibited fraction (>100%) and it is normal in resistant strains (WHO, 1998).

6.3 RESULTS

One-way ANOVA revealed that the mean of all tested enzyme activities in Malaysian *Cx. quinquefasciatus* were significantly different across all study sites ($P < 0.001$). In addition, Spearman rank-order correlation indicated a significant correlation between malathion survivability rate in adult bioassays and α-esterases activity in Malaysian *Culex quinquefasciatus* ($r = 0.634$; $P = 0.015$) (Figure 6.1), while no correlation was found with other insecticide survivability rates against others enzyme activities. Besides, an association between activity of α-esterases and β-esterases ($r = 0.570$; $P = 0.033$) and between glutathione-S-transferase and acetylcholinesterase ($r = 0.592$; $P = 0.026$) was also demonstrated (Figure 6.2).

In non-specific esterases microassay, the resistance ratios ranging from 1.09 to 2.04 fold and 1.0 to 1.62 fold for α-esterases activity and β-esterases activity, respectively, were recorded. A significant increase in α-esterases activity was detected in all populations (except Kelantan). A lack of elevated β-esterases activity was observed in Kelantan and Kedah populations, whereas other populations exhibited a

significant increase in β-esterases activity. All populations exhibited higher α-esterases activity, as compared to β-esterases activity (except Pahang) (Table 6.1).

As for mixed function oxidases microassay, the resistance ratios ranging from 0.84 to 2.16 fold were demonstrated. An elevated level of mixed function oxidases activity was found in nine populations (i.e., Kedah, Malacca, Negeri Sembilan, Penang, Perak, Sabah, Selangor, Sarawak and Terengganu) (Table 6.2).

The resistance ratios for glutathione-S-transferase microassay, ranging from 0.92 to 1.31 fold were recorded. Of 14 populations, nine populations (i.e., Kedah, Kelantan, Malacca, Pahang, Penang, Perak, Sabah, Sarawak and Terengganu) exhibited a significant increase in glutathione-S-transferase activity (Table 6.3).

With regard to insensitive acetylcholinesterase microassay, the resistance ratios ranging from 0.70 to 2.06 and 1.80 to 2.40 fold for control (uninhibited) and propoxur inhibition, respectively, were demonstrated. In control test, all populations revealed a significant increase in acetylcholinesterase activity (except Kuala Lumpur, Perlis and Sarawak). In comparison to the laboratory strain, all populations also revealed a significant increase in acetylcholinesterase activity after propoxur inhibition (Table 6.4). A quick perusal of the remaining activity data indicated that the RS was the most prevalent genotype in Malaysian *Cx. quinquefasciatus*, followed by SS genotype and RR genotype. An excess of RR genotype was recorded in *Cx. quinquefasciatus* population from Sarawak (Figure 6.3).

Summary of insecticide resistance and prevalence of resistance mechanisms in different *Cx. quinquefasciatus* populations was presented in Table 6.5. Elevated levels of all enzymes activities were demonstrated in four populations (Malacca, Penang, Perak and Terengganu).

Table 6.1 Mean non-specific esterases activity in Malaysian *Cx. quinquefasciatus* populations.

Strain	Mean ± SE (α-Na ηmol/min/mg protein)	RR	Mean ± SE (β-Na ηmol/min/mg protein)	RR
Reference	0.23 ± 0.01	-	0.21 ± 0.00	-
Kelantan	0.25 ± 0.01^{a}	1.09	0.21 ± 0.00^{a}	1.00
Terengganu	*0.29 ± 0.01^{abc}	1.26	*0.24 ± 0.01^{abc}	1.14
Pahang	*0.27 ± 0.00^{ab}	1.17	*0.32 ± 0.01^{ef}	1.39
Perlis	*0.32 ± 0.02^{bc}	1.39	*0.26 ± 0.01^{bcd}	1.13
Kedah	*0.29 ± 0.01^{abc}	1.26	0.21 ± 0.01^{a}	1.00
Penang	*0.32 ± 0.01^{bc}	1.39	*0.29 ± 0.00^{de}	1.38
Perak	*0.42 ± 0.02^{ef}	1.83	*0.34 ± 0.01^{f}	1.62
Selangor	*0.31 ± 0.01^{bc}	1.35	*0.24 ± 0.01^{ab}	1.14
Kuala Lumpur	*0.38 ± 0.01^{de}	1.65	*0.27 ± 0.00^{cd}	1.29
Negeri Sembilan	*0.34 ± 0.01^{cd}	1.48	*0.24 ± 0.01^{abc}	1.14
Malacca	*0.34 ± 0.01^{cd}	1.48	*0.26 ± 0.01^{bcd}	1.13
Johore	*0.30 ± 0.01^{abc}	1.30	*0.24 ± 0.00^{abc}	1.14
Sarawak	*0.28 ± 0.01^{ab}	1.22	*0.24 ± 0.00^{abc}	1.14
Sabah	*0.47 ± 0.02^{f}	2.04	*0.28 ± 0.00^{d}	1.33
One way ANOVA	***F* = 21.43; df = 13, 322; *P* < 0.0001**		***F* = 25.99; df = 13, 322; *P* < 0.0001**	

SE = standard error; RR = resistance ratio. Mean followed by a different letter were significantly different, $P < 0.05$, Tukey's test.

*Significant increase in mean differences compared to the laboratory reference strain, $P < 0.05$, t-test.

Table 6.2 Mean mixed function oxidases activity expressed at absorbance 630nm in Malaysian *Cx. quinquefasciatus* populations.

Strain	**Mean ± SE (Absorbance 630nm)**	**RR**
Reference	0.50 ± 0.02	-
Kelantan	0.53 ± 0.01[a]	1.06
Terengganu	*1.08 ± 0.05[e]	2.16
Pahang	0.42 ± 0.02[a]	0.84
Perlis	0.53 ± 0.02[a]	1.06
Kedah	*0.94 ± 0.05[de]	1.88
Penang	*0.93 ± 0.06[d]	1.86
Perak	*0.57 ± 0.02[ab]	1.14
Selangor	*0.83 ± 0.02[cd]	1.66
Kuala Lumpur	0.44 ± 0.01[a]	0.88
Negeri Sembilan	*0.84 ± 0.03[d]	1.68
Malacca	*0.96 ± 0.02[de]	1.92
Johore	0.53 ± 0.02[ab]	1.06
Sarawak	*0.87 ± 0.04[d]	1.74
Sabah	*0.68 ± 0.03[bc]	1.36
One way ANOVA	***F* = 46.98; df = 13, 322; *P* < 0.0001**	

SE = standard error; RR = resistance ratio. Mean followed by a different letter were significantly different, $P < 0.05$, Tukey's test.

*Significant increase in mean differences compared to the laboratory reference strain, $P < 0.05$, t-test.

Table 6.3 Mean glutathione-S-transferase activity in Malaysian *Cx. quinquefasciatus* populations.

Strain	**Mean ± SE (CDNA-ηmol/min/mg protein)**	**RR**
Reference	0.13 ± 0.00	-
Kelantan	*0.15 ±0.00^{def}	1.15
Terengganu	*0.15 ±0.00^{def}	1.15
Pahang	*0.16 ±0.00^{ef}	1.23
Perlis	0.13 ±0.01^{abc}	1.00
Kedah	*0.16 ±0.01^{f}	1.23
Penang	*0.17 ±0.00^{f}	1.31
Perak	*0.15 ±0.00^{def}	1.15
Selangor	0.12 ±0.00^{a}	0.92
Kuala Lumpur	0.12 ±0.00^{a}	0.92
Negeri Sembilan	0.14 ±0.00^{bcd}	1.08
Malacca	*0.14 ±0.00^{cde}	1.08
Johore	0.12 ±0.00^{ab}	0.92
Sarawak	*0.14 ±0.00^{cde}	1.08
Sabah	*0.14 ±0.00^{cde}	1.08
One way ANOVA	***F* = 18.75; df = 13, 322; *P* < 0.0001**	

SE = standard error; RR = resistance ratio. Mean followed by a different letter were significantly different, $P < 0.05$, Tukey's test.

*Significant increase in mean differences compared to the laboratory reference strain, $P < 0.05$, t-test.

Table 6.4 Mean acetylcholinesterase activity in control (uninhibited) and propoxur-inhibited fraction in Malaysian *Cx. quinquefasciatus* populations.

Strain	**Mean ± SE**	**RR**	**Mean ± SE**	**RR**
	Control (Without Insecticide)		**ACTH with 0.1% Propoxur**	
Reference	0.17 ± 0.01	-	0.05 ± 0.00	-
Kelantan	*0.22 ± 0.01^{bcd}	1.29	*0.09 ± 0.00^{a}	1.80
Terengganu	*0.32 ± 0.01^{fg}	1.88	*0.09 ± 0.00^{ab}	1.80
Pahang	*0.24 ± 0.01^{cde}	1.41	*0.09 ± 0.00^{ab}	1.80
Perlis	0.16 ± 0.01^{ab}	0.94	*0.09 ± 0.00^{ab}	1.80
Kedah	*0.35 ± 0.02^{g}	2.06	*0.10 ± 0.00^{ab}	2.00
Penang	*0.28 ± 0.01^{def}	1.65	*0.12 ± 0.00^{c}	2.40
Perak	*0.29 ± 0.02^{efg}	1.71	*0.09 ± 0.00^{ab}	1.80
Selangor	*0.24 ± 0.01^{cde}	1.41	*0.10 ± 0.00^{ab}	2.00
Kuala Lumpur	0.16 ± 0.01^{ab}	0.94	*0.09 ± 0.00^{a}	1.80
Negeri Sembilan	*0.24 ± 0.02^{cde}	1.41	*0.10 ± 0.00^{b}	2.00
Malacca	*0.21 ± 0.01^{bc}	1.24	*0.10 ± 0.00^{ab}	2.00
Johore	*0.20 ± 0.01^{bc}	1.18	*0.09 ± 0.00^{ab}	1.80
Sarawak	0.12 ± 0.00^{a}	0.71	*0.09 ± 0.00^{ab}	1.80
Sabah	*0.21 ± 0.01^{bc}	1.24	*0.10 ± 0.00^{b}	2.00
One way ANOVA	***F* = 22.25; df = 13, 322; *P* < 0.0001**		***F* = 7.65; df = 13, 322; *P* < 0.0001**	

SE = standard error; RR = resistance ratio. Mean followed by a different letter were significantly different, P < 0.05, Tukey's test.

*Significant increase in mean differences compared to the laboratory reference strain, *P* < 0.05, t-test.

Table 6.5 Summary of insecticide resistance and prevalence of resistance mechanisms in different *Cx. quinquefasciatus* populations in Malaysia.

	Insecticide Resistance				Elevated Enzyme Activity					
Strain	**DDT**	**PRO**	**MAL**	**PER**	**α-EST**	**β-EST**	**MFO**	**GST**	**AChE**	**pAChE**
Kelantan	R	R	M	R	−	−	−	+	+	+
Terengganu	R	R	S	M	+	+	+	+	+	+
Pahang	R	R	S	R	+	+	−	+	+	+
Perlis	R	R	R	R	+	+	−	−	−	+
Kedah	R	R	R	R	+	−	+	+	+	+
Penang	R	R	R	M	+	+	+	+	+	+
Perak	R	R	R	M	+	+	+	+	+	+
Selangor	R	R	R	R	+	+	+	−	+	+
Kuala Lumpur	R	R	R	R	+	+	−	−	−	+
Negeri Sembilan	R	R	R	S	+	+	+	−	+	+
Malacca	R	R	R	M	+	+	+	+	+	+
Johore	R	R	R	S	+	+	−	+	+	+
Sarawak	R	R	R	S	+	+	+	+	−	+
Sabah	R	R	R	M	+	+	+	−	+	+

*PRO = propoxur, MAL = malathion, PER = permethrin, α-EST = α-esterases, β-EST = β-esterases, MFO = mixed function oxidases, GST = glutathione-S-transferase, AChE = acetylcholinesterase, pAChE = propoxur-inhibited acetylcholinesterase, R = resistant, M = moderate resistant, S = susceptible, + = presence of mechanism, − = absence of mechanism

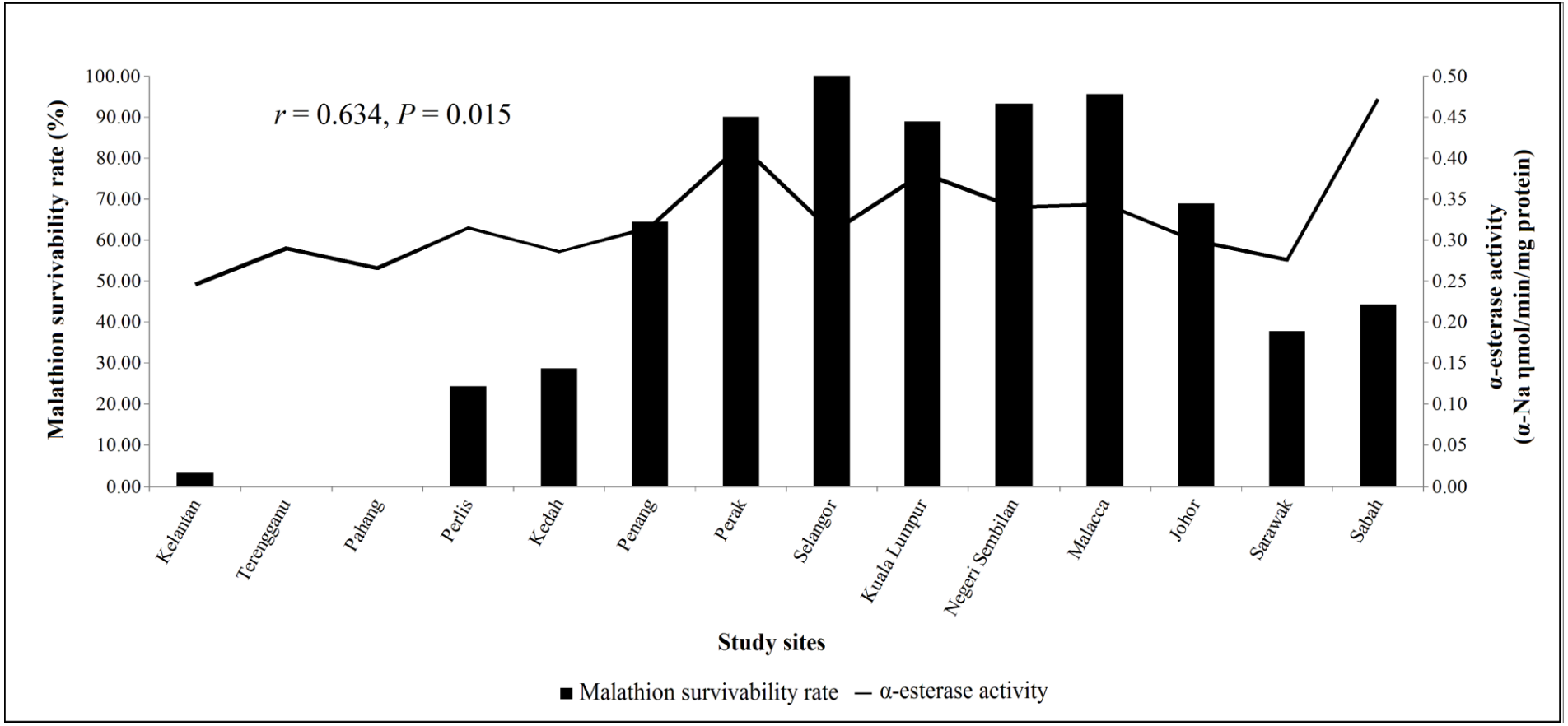

Figure 6.1 Spearman rank-order correlation between malathion survivability rate and α-esterases activity in Malaysian *Cx. quinquefasciatus*.

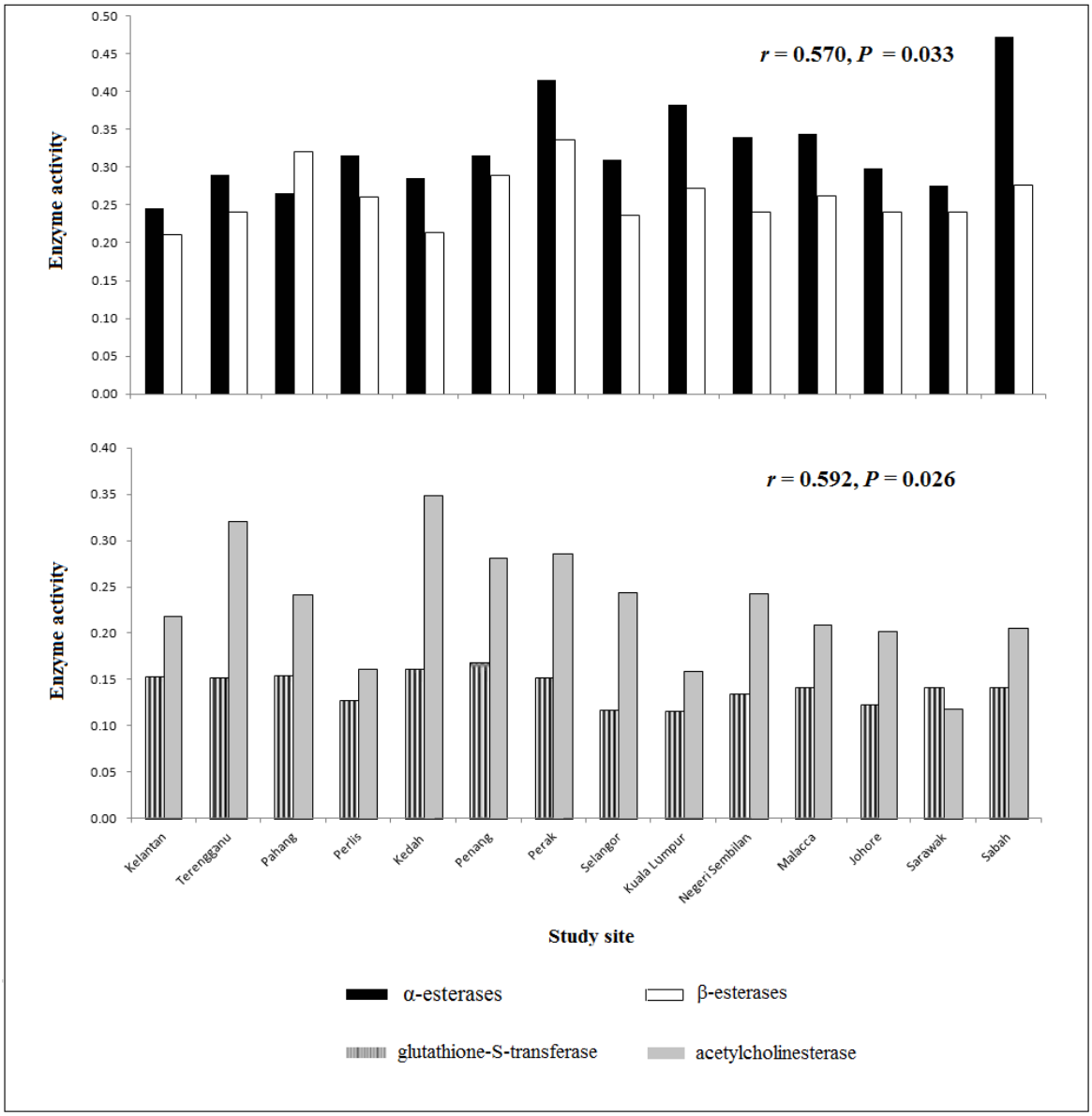

Figure 6.2 Spearman rank-order correlation between the activity of α-esterases and β-esterases and between glutathione-S-transferase and acetylcholinesterase in Malaysian *Cx. quinquefasciatus*.

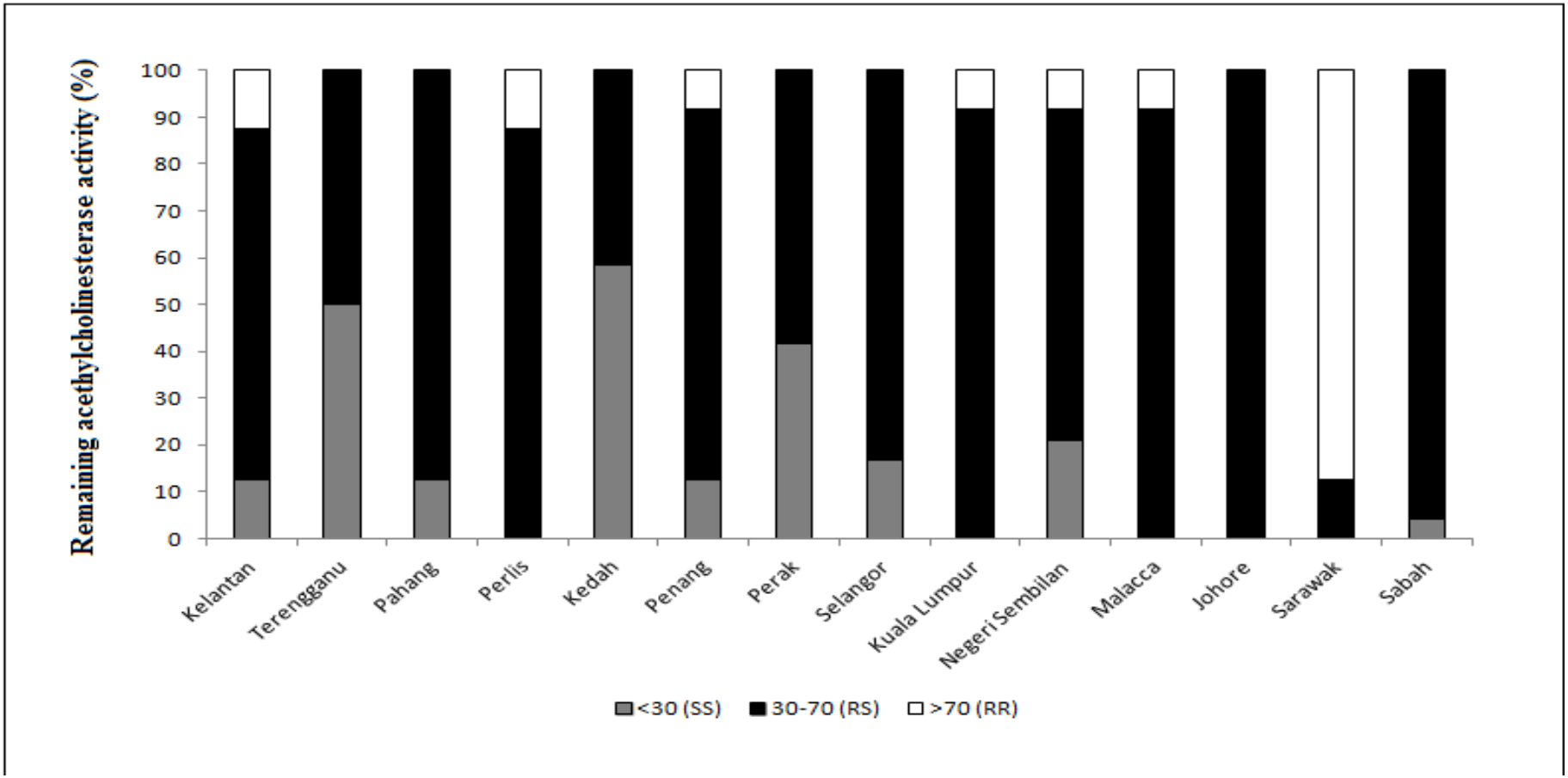

Figure 6.3 Percentage remaining activity of acetylcholinesterase in individual Malaysian *Cx. quinquefasciatus* after 0.1% propoxur inhibition.

* < 30% = homozygous susceptible (SS), 30-70% = heterozygous (RS), > 70% = homozygous resistance (RR), (WHO, 1998).

6.3 DISCUSSION

Regardless of the associations between the degree of insecticide resistance and the enzyme activities, the enhanced enzyme activities of α-esterases, β-esterases, mixed function oxidases, glutathione-S-transferase and insensitive acetylcholinesterase of Malaysian *Cx. quinquefasciatus* confirmed the incidence of insecticide resistance (low to high resistance towards DDT, propoxur, malathion and permethrin), as detected by WHO adult bioassays (refer Chapter 5). An elevated level of esterases (Lee, 1990; Lee *et al*., 1992; 1996, Nazni *et al*., 1998) and oxidases (Nazni *et al*., 2000; 2004) in field populations of Malaysian *Cx. quinquefasciatus* has been previously described. However, the elevated level of glutathione-S-transferase and acetylcholinesterase in this study indicated contrasting results with previous studies, where there were lacks of elevated levels of glutathione-S-transferase and acetylcholinesterase after propoxur inhibition in Malaysian *Cx. quinquefasciatus* (Lee & Chong, 1995; Nazni *et al*., 2004).

Statistical analysis demonstrated that there was a significant association between malathion survivability rate in adult bioassays and α-esterases activity in Malaysian *Culex quinquefasciatus*, suggested that the development of malathion resistance in these populations was due to the increased levels of α-esterases activity. Elevated esterases levels associated with organophosphate resistance in Malaysian *Cx. quinquefasciatus* has also been reported in earlier studies (Lee, 1990; Lee *et al*., 1992). Likewise, similar detoxification mechanism has also been found in Malaysian *Aedes aegypti* (Chen *et al*., 2008). In addition, an association between increased levels of esterases and carbamate resistance has been documented in cockroach *Blattella germanica* from Malaysia (Lee *et al*., 2000). In Malaysia, α-esterases in Malaysian mosquitoes have been the focus of study but not β-esterases. However, a study in Malaysia has investigated both enzymes in cockroach *Blattella germanica* and indicated a higher activity of β-esterases, as

compared to α-esterases (Lee *et al*., 2000). In contrast, the present study indicated that activity of α-esterases was higher than β-esterases in all populations (except Pahang). Similar observation has also been found in *Ae. aegypti* and *Ae. albopictus* populations in Thailand (Pethuan *et al*., 2007). Meanwhile, inconsistent trends in both α-esterases and β-esterases activities have been demonstrated in Indian *Cx. quinquefasciatus* populations (Sarkar *et al*., 2009a).

With regard to other insecticide survivability rates against any other enzyme activities, no correlation was found in the present study. However, previous studies reported that elevated levels of oxidases were correlated with pyrethroid resistance in Malaysian *Ae. albopictus* (Wan-Norafikah *et al*., 2008) and *Ae. Aegypti* (Wan-Norafikah *et al*., 2010). Although glutathione-S-transferase is responsible for resistance to various insecticide classes across a number of insect species, it has primarily been associated with DDT resistance (Hollingworth & Dong, 2008). Earlier study has attempted to establish the correlation between glutathione-S-transferase activity and DDT resistance in Malaysian *Anopheles maculatus*, *Cx. quinquefasciatus* and *Ae. aegypti*, but failed to demonstrate a clear relationship (Lee & Chong, 1995). Even today, the associations between glutathione-S-transferase activity and insecticide resistance in Malaysian mosquitoes were poorly evidenced. On the other hand, it is important to emphasize that DDT has not been applied in Malaysia since 1998, but the resistance phenotype still remains in Malaysian populations. It certainly does not exclude the consequence of the extensive use of pyrethroids in recent years which also conferred resistance to DDT, as both pyrethroids and DDT are specifically designed to attack the voltage-gated sodium channel of insect (Hemingway *et al*., 2004). As for insensitive acetylcholinesterase, its activity in Malaysian *Cx. quinquefasciatus* has been sensitive to propoxur (Lee *et al*., 1992). However, the present results have provided strong evidence on the role of insensitive acetylcholinesterase in the development of propoxur resistance

in Malaysian *Cx. quinquefasciatus*. Besides, organophosphate resistance conferred by insensitive acetylcholinesterase has also been observed in Malaysian *Blattella germanica* (Lee *et al*., 1997b).

Multiple insecticide resistance mechanisms imply that more than one mechanism is involved in insecticide resistance. In fact, multiple insecticide resistance mechanisms are not new phenomenon and are becoming problematic worldwide. In the present study, an association between activity of α-esterases and β-esterases and between glutathione-S-transferase and insensitive acetylcholinesterase were demonstrated. The association between the activity of α-esterases and β-esterases has also been documented previously (Norris & Norris, 2011) and it is proposed that the occurrence of this incidence might be due to the co-amplification of two esterase genes (est$\alpha 2^1$ and est$\beta 2^1$) which was commonly found in organophosphate-resistant *Cx. quinquefasciatus* (Hemingway *et al*., 2004). Meanwhile, selection of carbamate in western flower thrips, *Frankliniella occidentalis* populations has demonstrated that an increased level of resistance resulted in elevated activities of acetylcholinesterase and glutathione-S-transferase (Jensen, 2000). It has been suggested that both elevated levels of acetylcholinesterase and glutathione-S-transferase activities could contribute to carbamate resistance.

In conclusion, the results presented here provide the first report on the roles of α-esterases, β-esterases, mixed function oxidases, glutathione-S-transferase and acetylcholinesterase towards resistance to DDT, propoxur, malathion and permethrin in *Cx. quinquefasciatus* from all states in Malaysia. Evidence of malathion resistance due to elevated α-esterases activity was found. In addition, an association between activity of α-esterases and β-esterases and between glutathione-S-transferase and insensitive acetylcholinesterase was also demonstrated in the present study.

CHAPTER 7

MOLECULAR CHARACTERIZATION OF INSECTICIDE RESISTANCE MECHANISMS IN MALAYSIAN *CULEX QUINQUEFASCIATUS*

7.1 INTRODUCTION

Extensive use of insecticides for vector-borne disease control has contributed to insecticide resistance development in the target species (WHO, 2006). In Malaysia, susceptibility of mosquitoes against various insecticides has been studied extensively and described by various approaches such as WHO larval and adult bioassays (Reid, 1955; Thomas, 1962; Lee *et al*., 1997a; Chen *et al*., 2005b; Nazni *et al*., 2005; Hidayati *et al*., 2011), enzyme microassays (Lee, 1990; Nazni *et al*., 2000; Selvi *et al*., 2007; 2010) as well as protein electrophoresis (Selvi *et al*., 2010). However, so far nothing has been reported pertaining to insecticide resistance gene detection at a molecular level. There is a dearth of evidence of insecticide resistance in Malaysian mosquitoes on molecular basis.

In the last few decades, organochlorine insecticides (i.e., DDT) have been heavily used in pest control programs (Whalon *et al*., 2008). However, while the ultimate or progressively evolving DDT resistance in insect pests were documented, in recent decades, pyrethroid-based insecticides have been introduced as alternatives to DDT (Chavasse & Yap, 1997). Both pyrethroids and DDT attack the voltage gated sodium channel of insects leading to the development of 'knockdown resistance' when there is an excessive use of either class of insecticide (Hemingway *et al*., 2004). On the other hand, an elevated level of esterase activity has been identified to play a key role in

organophosphate and carbamate resistance development (Lee, 1990; Nazni *et al*., 2000; Selvi *et al*., 2007). Additionally, numerous studies have also reported that mutation in acetylcholinesterase target site is the main factor conferring resistance in organophosphates and carbamates (Hemingway *et al*., 2004).

Particularly, insecticide resistance in the Malaysian *Cx. quinquefasciatus* has been well-observed. Over the years, insecticide resistance towards DDT, pyrethroids, carbamates and organophosphates in Malaysian *Cx. quinquefasciatus* has been reported (Reid, 1955; Lee *et al*., 1997a; Nazni *et al*., 2000; 2005; Selvi *et al*., 2007; Hidayati *et al*., 2011). However, in Malaysia, considerable research efforts have focused mainly on biochemical characterization of enzyme-based metabolic mechanisms (Lee, 1990; Nazni *et al*., 2000; Selvi *et al*., 2007). Indeed, there is a lack of evidence of insecticide resistance conferred by mutations in the voltage gated sodium channel and acetylcholinesterase in Malaysian mosquitoes as well as other insect species in Malaysia.

Based on previous report (refer Chapter 5), Malaysian *Cx. quinquefasciatus* populations have developed a wide spectrum of insecticide resistance towards DDT, propoxur, malathion and permethrin, as demonstrated by WHO larval and adult bioassays. Hence, this study was carried out to further confirm the incidences of resistance that were detected in previous study, thereby attempting to investigate the prevalence of the *kdr* and *ace-1*^{R} mutations in *Cx. quinquefasciatus* populations from 11 states and a federal territory (i.e., Kuala Lumpur) in Peninsular Malaysia as well as two states in East Malaysia. In addition to providing a better understanding of evolutionary relationship in this mosquito species, the information gathered from this study could improve the knowledge of vector control and management in Malaysia.

7.2 MATERIALS AND METHODS

7.2.1 MOSQUITO STRAINS

In the present study, a total of 140 adult *Cx. quinquefasciatus* with 10 individual mosquitoes representing each of the 14 study sites were randomly selected.

7.2.2 DNA EXTRACTION

Prior to DNA extraction**,** abdomens were dissected from mosquito samples to avoid contamination. DNA was extracted from each specimen using i-genomic CTB DNA Extraction Mini Kit^{TM} (iNtRON Biotechnology, Inc, Korea). All isolation steps were performed according to manufacturer instructions (Refer Appendix A).

7.2.3 DETECTION OF VOLTAGE GATED SODIUM CHANNEL MUTATION

7.2.3.1 ALLELE-SPECIFIC (AS)-PCR METHOD

A modified three tube AS-PCR method (Martinez-Torres *et al*., 1999; Wang *et al*., 2012) was performed to detect the presence of 1014F and 1014S alleles (Figure 7.1). Three separate PCR reactions were conducted by using the mixture of CD1 primer, 5'-AAC TTC ACC GAC TTC ATG CAC-3' and CD2 primer, 5'-CAA GGC TAA GAA AAG GTT AAG AAC-3' with CD3 specific primer, 5'-CCA CCG TAG TGA TAG GAA ATT TA-3' for the TTA (Leu) detection, CD4 specific primer, 5'-CCA CCG TAG TGA TAG GAA ATT TT-3' for the TTT (Phe) detection or CD5 specific primer, 5'-

CCA CCG TAG TGA TAG GAA ATT C-3’ for the TCA (Ser) detection. The ratio of the primer mixture was CD1 : CD2 : CD3/4/5 = 3 : 10 : 7.

The amplification of sodium channel region was performed in a final volume of 25μl containing 25-50ng genomic DNA of mosquito, 12μl of ExPrime Taq Master Mix™ (GENET BIO, Korea) and 2μl of primer mixture. PCR was carried out using Bio-rad MyCycler™ Thermal Cycler Serial Number: 580BR 7200 (CA, USA). The PCR conditions included an initial denaturation of 94℃ for 2min, followed by 35 cycles of 94℃ for 30s (denaturation), 60℃ for 30s (annealing), 72℃ for 45s (extension) and a final extension at 72℃ for 10min (Wang *et al*., 2012). The amplified fragments were electrophoresed on 2% agarose gel pre-stained with SYBR safe™ (Invitrogen, USA) in TAE buffer.

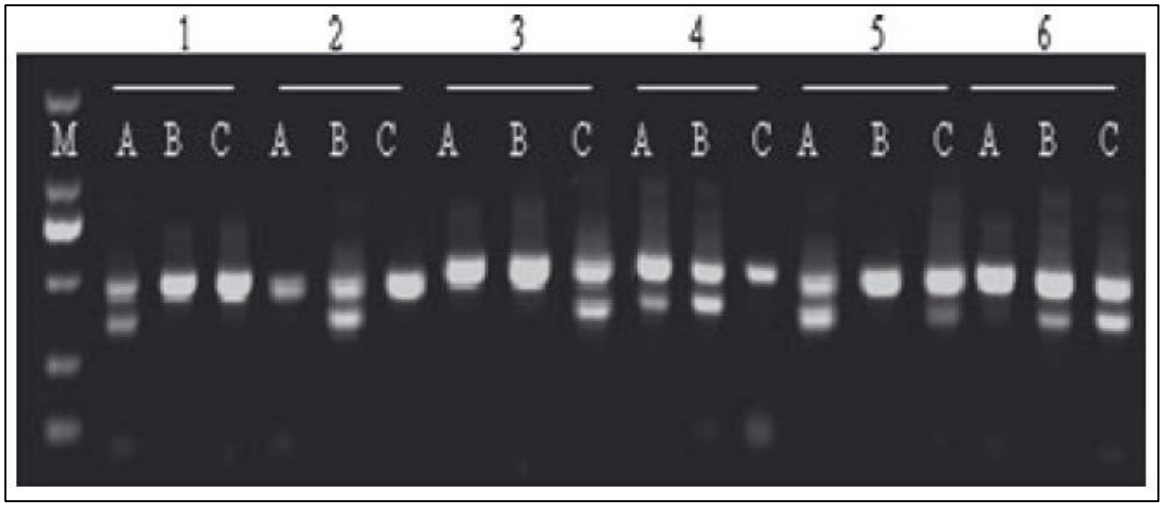

Figure 7.1 Detection of L1014 mutation by AS-PCR method. 1-6: the number of the sample. (A) contained the special primer CD3 and signified the 1014L genotype; (B) contained the special primer CD4 and signified the 1014F genotype; (C) contained the special primer CD5 and signified the 1014S genotype.

(Wang *et al*., 2012)

7.2.3.2 DIRECT SEQUENCING METHOD

A subset of 40 individual samples was screened for *kdr* mutation by direct sequencing. New primers were designed based on cloned sequences (KC189872 and KC189873): JKDR_F, forward primer, 5'-GGA TCG AAT CCA TGT GGG ACT-3' and JKDR_R, reverse primer, 5'-TGC ACC TTT AGG TGT GGA CCT TC-3'.

The amplification of sodium channel region was performed in a final volume of 50µl containing 5µl 10x buffer, 2.5mM of each dNTP, 10pmol of each forward and reverse primer, 1.5U *Taq* polymerase (iNtRON Biotechnology, Inc, Korea) and 25-50ng genomic DNA of mosquito. PCR was carried out using Bio-rad MyCycle™ Thermal Cycler Serial Number: 580BR 7200 (CA, USA). The PCR conditions included an initial denaturation of 94℃ for 5min, followed by 40 cycles of 94℃ for 45s (denaturation), 59℃ for 45s (annealing), 72℃ for 45s (extension) and a final extension at 72℃ for 5min.

The amplified fragments (~285bp) were electrophoresed on 2% agarose gel pre-stained with SYBR Safe™ (Invitrogen, USA) in TAE buffer. The PCR products were purified with MEGAquick-spin™ PCR & Agarose Gel DNA Extraction System (iNtRON Biotechnology, Inc, Korea) (refer Appendix B).

The purified PCR products were sent to a commercial company for DNA sequencing in both directions. Samples were sequenced using BigDyeH Terminator v3.1 Sequencing Kit™ and analyzed on ABI PRISMH 377 Genetic Analyzer™.

Sequencing data were analyzed and edited using ChromasPro 1.5® (Technelysium Pty Ltd., Australia) and BioEdit 7.0.9.0.® (Hall, 1999). The sodium channel sequences were preliminarily aligned using the CLUSTAL X® program (Thompson *et al*., 1997) and subsequently aligned manually. Heterogenous mutations were quantified based on both forward and reverse sequences where the heterozygous

genotype (RS) exhibited double peaks in the mutation point, whereas homozygous genotype (RR/SS) exhibited only one specific peak (Simsek *et al*., 2001). (Figure 7.2). Representative sequences of the sodium channel gene of *Cx. quinquefasciatus* in this study were deposited in GenBank under the accession numbers KC189872-KC189889.

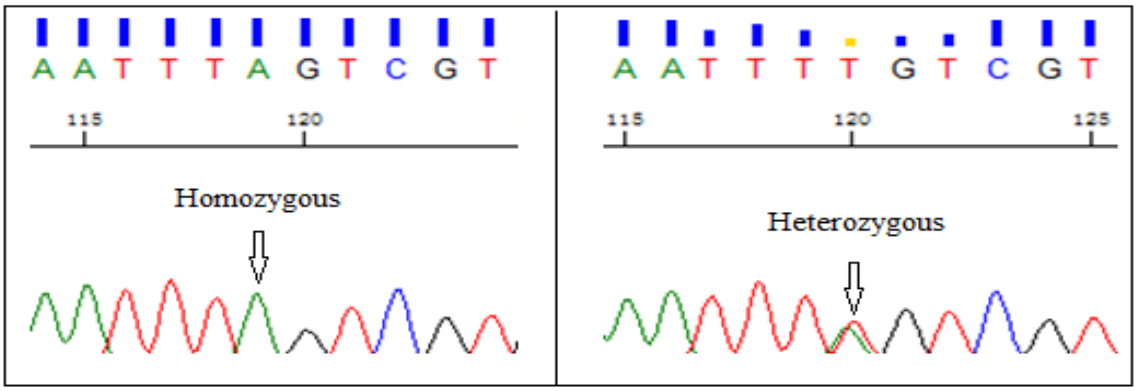

Figure 7.2 Heterogenous point mutations in voltage gated sodium channel sequences of *Cx. quinquefasciatus*.

7.2.4 DETECTION OF ACETYLCHOLINESTERASE MUTATION

7.2.4.1 DIRECT SEQUENCING METHOD

The amplification of extracted genomic DNA was conducted using primers of *ace-1* from Cui *et al*. (2006) : forward primer, 5'-CGA CTC GGA CCC ACT GGT-3' and reverse primer, 5'-GTT CTG ATC AAA CAG CCC CGC-3'. The amplification of *ace-1* region was performed in a final volume of 50 µl containing 5 µl 10x buffer, 2.5mM of each dNTP, 10pmol of each forward and reverse primer, 1.5U *Taq* polymerase (iNtRON Biotechnology, Inc, Korea), 25-50ng genomic DNA of mosquito. PCR was carried out using Bio-rad MyCycler^{TM} Thermal Cycler Serial Number: 580BR 7200 (CA, USA). The PCR conditions of *ace-1* included an initial denaturation of 94 ℃ for

5min, followed by 30 cycles of 94 ℃ for 30s (denaturation), 57 ℃ for 30s (annealing), 72 ℃ for 1min (extension) and a final extension at 72 ℃ for 5min.

A total of 140 purified PCR products were sent to a commercial company for DNA sequencing in both directions. Samples were sequenced using BigDyeH Terminator v3.1 Sequencing Kit™ and analyzed on ABI PRISMH 377 Genetic Analyzer™.

Sequencing data were analyzed and edited using ChromasPro 1.5® (Technelysium Pty Ltd., Australia) and BioEdit 7.0.9.0.® (Hall, 1999) The *ace-1* sequences were preliminarily aligned using the CLUSTAL X® program (Thompson *et al*., 1997) and subsequently aligned manually. Heterogenous mutations were quantified on the basis of both forward and reverse sequences where the heterozygous genotype (RS) exhibited double peaks in the mutation point, whereas homozygous genotype (RR/SS) exhibited only one specific peak (Simsek *et al*., 2001) (Figure 7.3). Representative sequences of the *ace-1* gene of *Cx. quinquefasciatus* in this study were deposited in GenBank under the accession numbers JX575102 to JX575112.

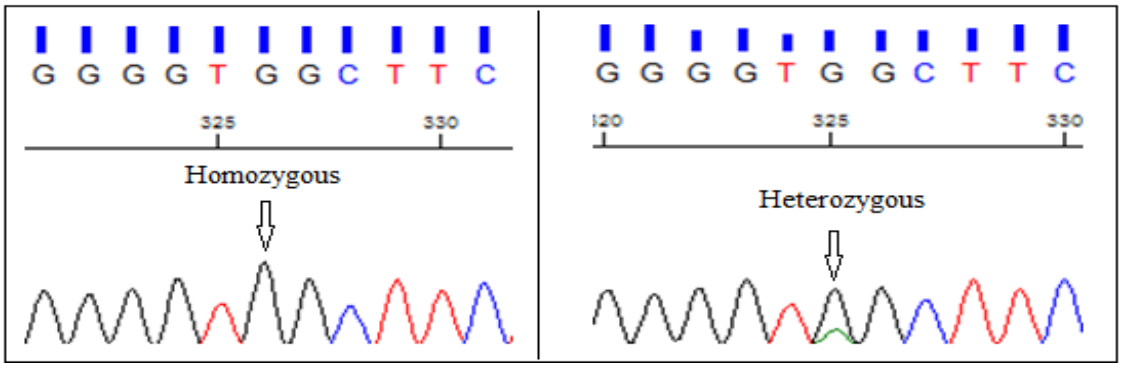

Figure 7.3 Heterogenous point mutations in acetylcholinesterase sequences of *Cx. quinquefasciatus*.

7.2.4.2 PCR-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) METHOD

A subset of 70 samples (including the 18 samples that exhibited RS genotype by sequencing) was subjected to PCR-RFLP. The PCR fragments were digested with 1µl of FastDigest *Alu* I (Thermo Fisher Scientific, Inc, USA) for 15min and fractionated on a 2% agarose gel pre-stained with SYBR Safe™ (Invitrogen, USA).

The two primers produced a fragment, which is undigested by *Alu*I for SS genotype and cut into two fragments for RR genotype. On the other hand, RS genotype exhibits a combined pattern (Weill *et al*., 2004; Cui *et al*., 2006) (Figure 7.4).

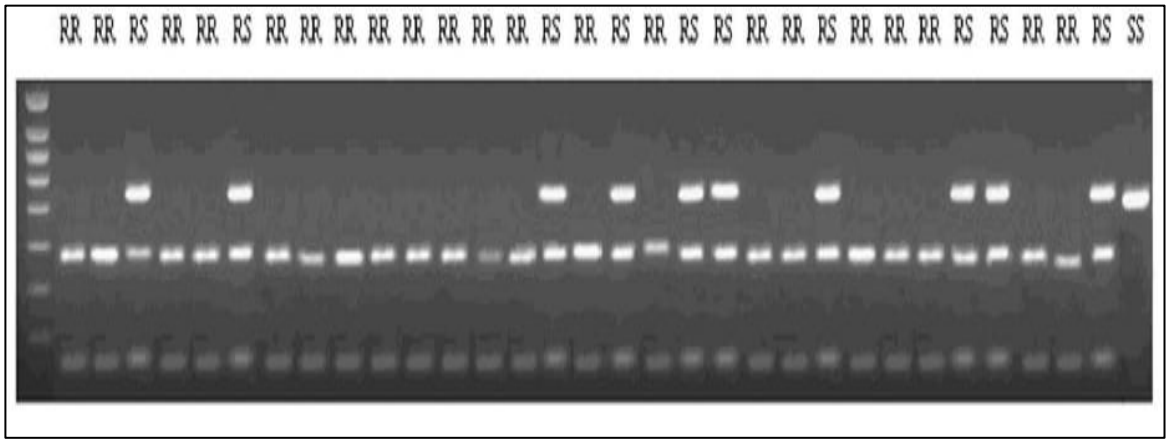

Figure 7.4 Detection of G119S mutation by PCR-RFLP method. One fragment = SS genotype; two fragments = RR genotype; three fragments = RS genotype.

(Cui *et al*., 2006)

7.2.5 STATISTICAL ANALYSIS

The frequencies of *kdr* and *ace-1* alleles were determined by Hardy-Weinberg Equilibrium, using GenePOP® (version 3.4) software (Raymond & Rousset, 1995).

7.3 RESULTS

The AS-PCR method demonstrated the presence of the classical 1014F mutation in all of the wild populations of *Cx. quinquefasciatus*, while the 1014S mutation was not detected across all study sites in Malaysia (Figure 7.5 and Table 7.1). Overall, the SS genotype was found in a majority of the study sites (9 out of 14) with 38 individuals from a total sample size of 140. It is of interest that the RS genotype was detected across all study sites and was most predominant with 99 individuals from a total sample size of 140. Of 14 populations, two populations (i.e., Perak and Selangor) indicated the presence RR genotype but at very low frequencies (3 individuals).

The genotype frequencies at *kdr* locus from seven populations (i.e., Johore, Kedah, Kelantan, Sabah, Sarawak, Selangor and Terengganu) conformed to the Hardy-Weinberg expectations at the 95% confidence level ($P > 0.05$). Inversely, the genotype frequencies at *kdr* locus from another seven populations (i.e., Kuala Lumpur, Malacca, Negeri Sembilan, Pahang, Penang, Perak and Perlis) differed significantly ($P \leq 0.05$). The resistance *kdr* allele frequencies ranged from 0.1 to 0.55, with the highest being detected in *Cx. quinquefasciatus* population from Selangor (Table 7.1).

The results of DNA sequencing of 40 individual samples also revealed the presence of 1014F mutation, while no other mutations were detected. Of these 40 individual samples, 24 were assigned as SS genotype, 13 as RS genotype and 3 as RR genotype. However, the results of DNA sequencing were not in complete agreement with AS-PCR method (Table 7.2).

No association was found between the frequencies of *kdr* resistant allele and the DDT and pyrethroids resistance phenotype.

Table 7.1 Genotypes and frequency of *kdr* alleles in Malaysian *Cx. quinquefasciatus*.

		Genotype			Allele Frequency		HW
Localities	**n**	**SS**	**RS**	**RR**	**S**	**R**	**(p-value)***
Kelantan	10	6	4	0	0.80	0.20	1.00
Terengganu	10	2	8	0	0.60	0.40	0.17
Pahang	10	1	9	0	0.55	0.45	0.05
Perlis	10	0	10	0	0.50	0.50	0.01
Kedah	10	8	2	0	0.90	0.10	1.00
Penang	10	0	10	0	0.50	0.50	0.01
Perak	10	9	0	1	0.90	0.10	0.05
Selangor	10	1	7	2	0.45	0.55	0.52
Kuala Lumpur	10	0	10	0	0.50	0.50	0.01
Negeri Sembilan	10	0	10	0	0.50	0.50	0.01
Malacca	10	0	10	0	0.50	0.50	0.01
Johore	10	6	4	0	0.80	0.20	1.00
Sarawak	10	3	7	0	0.65	0.35	0.22
Sabah	10	2	8	0	0.60	0.40	0.17
Total	**140**	**38**	**99**	**3**	**0.63**	**0.37**	**0.00**

HW = Hardy-Weinberg test.

*The exact probability for rejecting Hardy-Weinberg equilibrium.

Table 7.2 *kdr* genotypes detected by both AS-PCR and sequencing methods.

N	AS-PCR			Sequencing		
	TTA (SS)	TTA/T (RS)	TTT (RR)	TTA (SS)	TTA/T (RS)	TTT (RR)
40	21	16	3	24	13	3

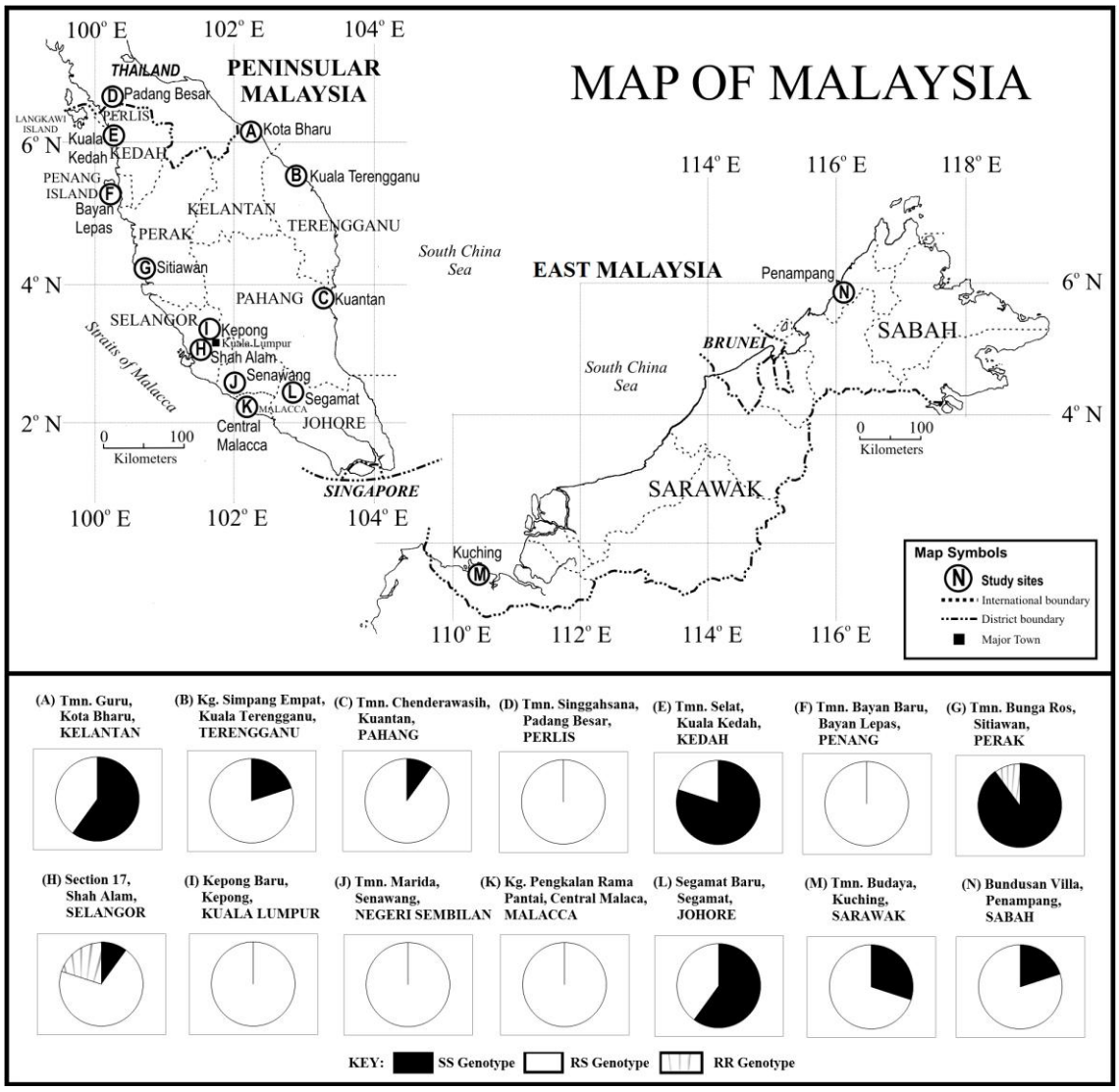

Figure 7.5 Genotype distribution of *kdr* gene in *Cx. quinquefasciatus* across all study sites in Malaysia.

As for insensitive acetylcholinesterase, both sequencing and PCR-RFLP methods exhibited similar results and confirmed the presence of glycine-serine *ace-1* mutation in the wild population of *Cx. quinquefasciatus* (Figure 7.6 and Table 7.3). Overall, the SS genotype was found in all 14 locations and was the most predominant with 122 individuals from a total sample size of 140, followed by RS genotype (18 individuals), while no RR genotype was detected across all states in Malaysia. Out of 14 populations, seven populations (i.e., Penang, Perak, Selangor, Kuala Lumpur, Negeri Sembilan, Malacca and Sabah) exhibited the G119S mutation, but only at heterozygote state. Within these seven populations, the genotype frequencies were not significantly different from Hardy-Weinberg expectations at the 95% confidence level ($P > 0.05$). The *ace-1*^{R} allele was most widespread in *Cx. quinquefasciatus* from Sabah (*ace-1*^{R} allele frequency = 0.30). Spearman rank-order correlation revealed that there was a significant correlation between the malathion resistance ratio and frequency of *ace-1*^{R} allele ($r = 0.543$, $P = 0.045$). In addition, in adult stage, a significant correlation was also detected between the frequency of *ace-1*^{R} allele and malathion survivability rate ($r = 0.653$, $P = 0.011$) (Figure 7.7). With regard to propoxur, no correlation was detected at either the larval or the adult stage.

Co-inheritance of L1014F and G119S mutations in individual *Cx. quinquefasciatus* was detected in seven populations (i.e., Kuala Lumpur, Malacca, Negeri Sembilan, Penang, Perak, Sabah and Selangor) but at a very low frequency (12 out of 140 individuals) (Table 7.4).

Table 7.3 Genotypes and frequency of *ace-1* alleles in Malaysian *Cx. quinquefasciatus*.

		Genotype			Allele Frequency		HW
Localities	**n**	**SS**	**RS**	**RR**	**S**	**R**	**(*P*-value)***
Kelantan	10	10	0	0	1.00	0.00	0.00
Terengganu	10	10	0	0	1.00	0.00	0.00
Pahang	10	10	0	0	1.00	0.00	0.00
Perlis	10	10	0	0	1.00	0.00	0.00
Kedah	10	10	0	0	1.00	0.00	0.00
Penang	10	8	2	0	0.90	0.10	1.00
Perak	10	8	2	0	0.90	0.10	1.00
Selangor	10	9	1	0	0.95	0.05	1.00
Kuala Lumpur	10	9	1	0	0.95	0.05	1.00
Negeri Sembilan	10	5	5	0	0.75	0.25	1.00
Malacca	10	9	1	0	0.95	0.05	1.00
Johore	10	10	0	0	1.00	0.00	0.00
Sarawak	10	10	0	0	1.00	0.00	0.00
Sabah	10	4	6	0	0.70	0.30	0.48
Total	**140**	**122**	**18**	**0**	**0.94**	**0.06**	**1.00**

HW = Hardy-Weinberg test.

*The exact probability for rejecting Hardy-Weinberg equilibrium.

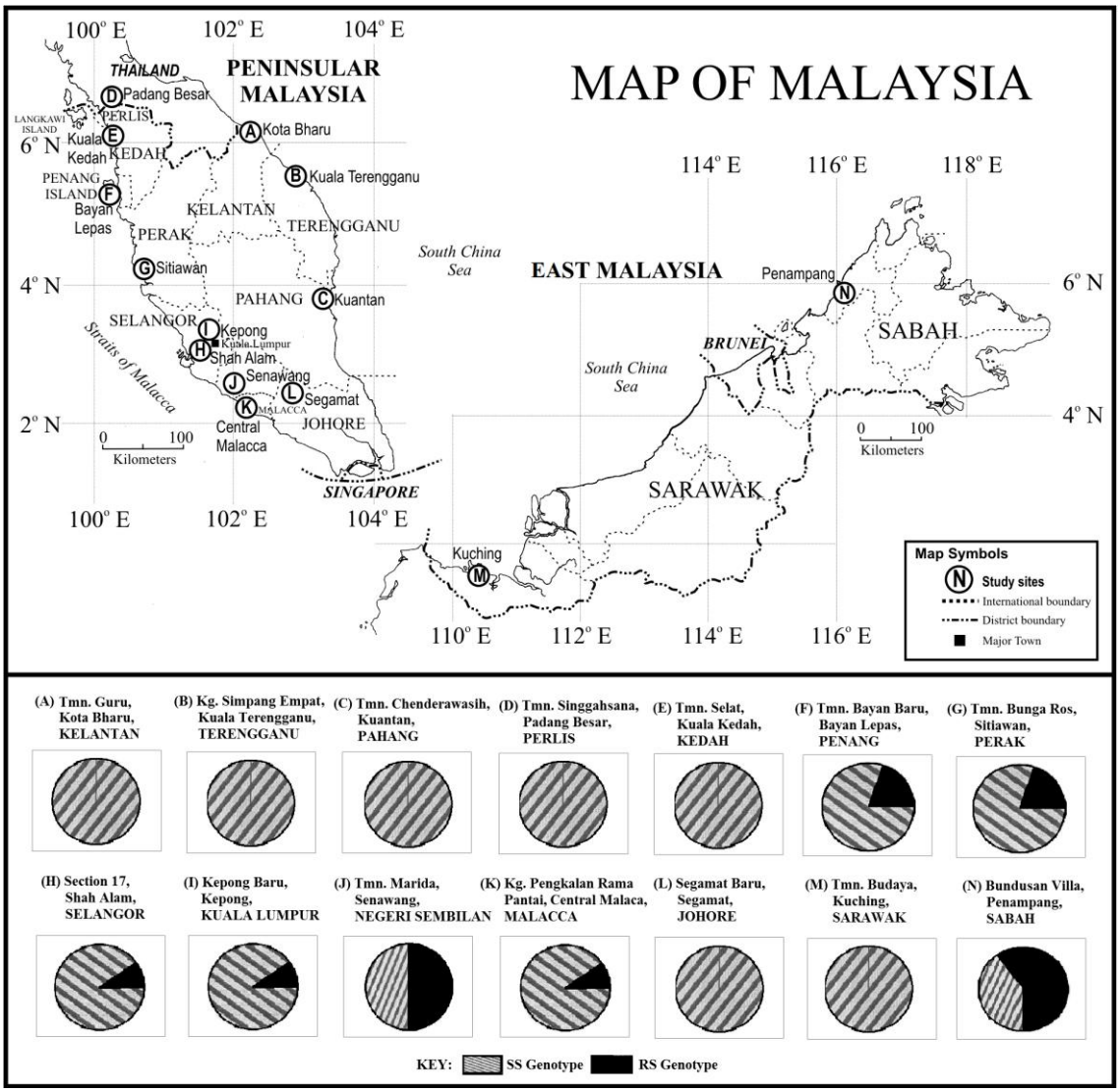

Figure 7.6 Genotype distribution of *ace-1* gene in *Cx. quinquefasciatus* across all study sites in Malaysia.

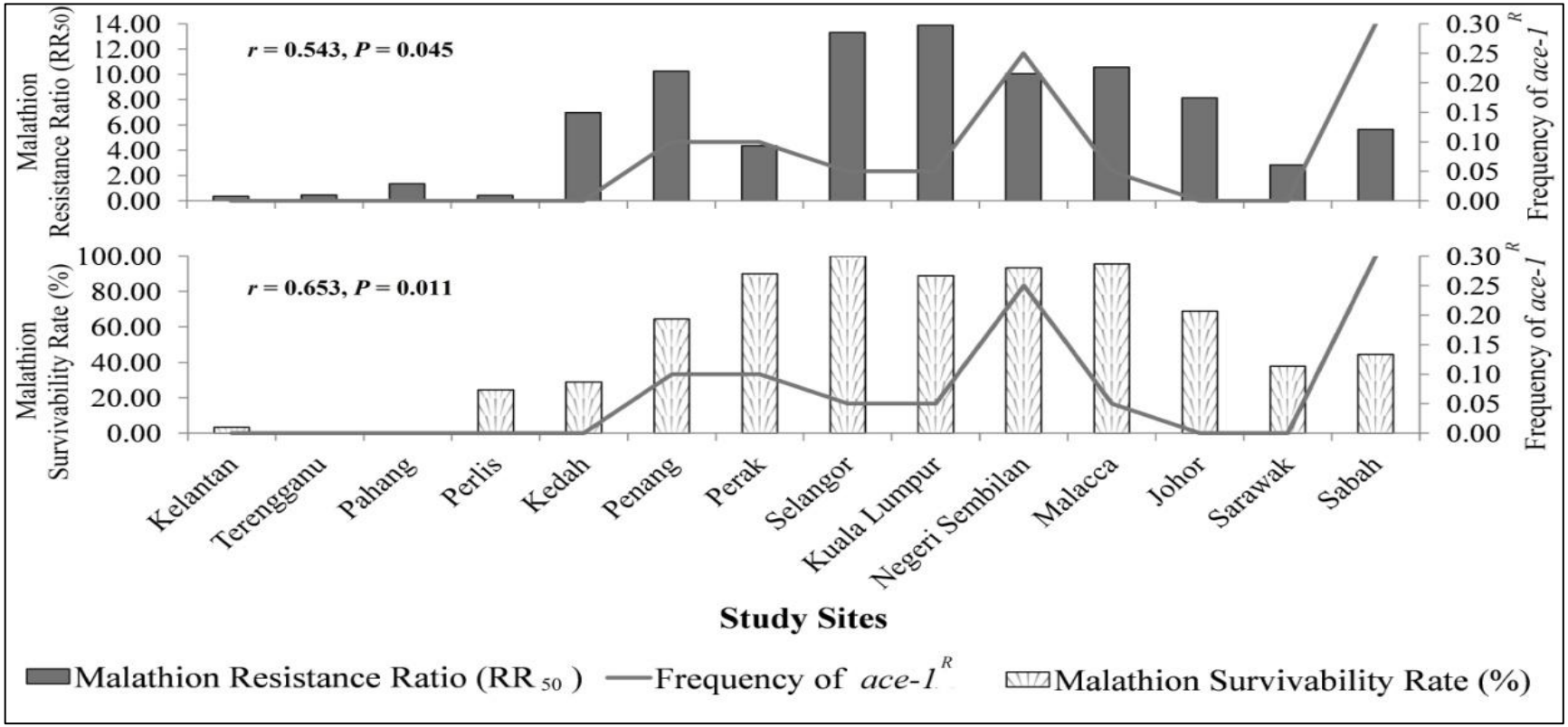

Figure 7.7 Spearman rank-order correlation between the frequency of *ace-1*^{R} with malathion resistance ratio (larval stage) and malathion survivability (adult stage).

Table 7.4 Co-inheritance of L1014F and G119S mutations in Malaysian *Cx. quinquefasciatus*.

Localities	n	Co-inheritance of G119S and L1014F (n)
Kelantan	10	0
Terengganu	10	0
Pahang	10	0
Perlis	10	0
Kedah	10	0
Penang	10	1
Perak	10	1
Selangor	10	1
Kuala Lumpur	10	1
Negeri Sembilan	10	2
Malacca	10	1
Johore	10	0
Sarawak	10	0
Sabah	10	5
Total	**140**	**12**

7.4 DISCUSSION

The distribution of 1014 mutation(s) in *Cx. quinquefasciatus*, at varying frequencies has been reported worldwide (Liu *et al*., 2009; Sarkar *et al*., 2009b; Zhou *et al*., 2009; Wang *et al*., 2012; Jones *et al*., 2012). In the current study, the classical knockdown resistance, L1014F mutation (TTA to TTT) at varying frequencies was detected from all populations, while the L1014S mutation (TTA to TCA) and other mutations reported previously were not detected in Malaysian *Cx. quinquefasciatus*. It has been documented that mosquitoes with L1014F mutation contributed high levels of resistance against both DDT and pyrethroids, while the L1014S mutation contributed high levels of resistance against DDT but low levels of resistance against pyrethroids (Martinez-Torres *et al*., 1999). Based on previous report, the Malaysian *Cx. quinquefasciatus* populations displayed high levels of resistance against DDT but relatively low levels of resistance (or susceptible) against permethrin. It is suspected that the widespread L1014F mutation occurring in Malaysian *Cx. quinquefasciatus* has resulted in the development of high DDT resistance. Likewise, a recent study also indicated that Indian *Cx. quinquefasciatus* with L1014F mutation demonstrated high DDT resistance but was susceptible against deltamethrin (Sarkar *et al*., 2009b). However, this study does not exclude the involvement of metabolic mechanisms which can occur in the same population, as observed by Djouaka *et al*. (2008).

Attempts to determine the relationship between the frequency of *kdr* resistance allele with the insecticide susceptibility status in both larval and adult stages were made, but no association was found in both stages with regard to DDT and permethrin. Previous studies elsewhere have reported different relationship between the frequencies of *kdr* resistant allele and the DDT and pyrethroids resistance phenotype. Insecticide resistance phenotype in several species of mosquito, house flies as well as cockroach

was found to be correlated with the frequencies of *kdr* resistant allele (Miyazaki *et al*., 1996; Williamson *et al*., 1996; Martinez-Torres *et al*., 1998; Liu *et al*., 2009; Wang *et al*., 2012). Inversely, a number of studies also reported that no association between the *kdr* mutation and insecticide resistance phenotype in other insect species (Dong *et al*., 1998; Yawson *et al*., 2004; Xu *et al*., 2005; Sarkar *et al*., 2009b). Given the lack of this association, it is possible that multiple resistance mechanisms involving both target site alterations and detoxification activities could be occurred.

There have been many arguments about the accuracy of both PCR and sequencing methods for the detection of heterozygous in an individual sample (Simsek *et al*., 2001). In the present study, it was found that the results of DNA sequencing were not in agreement with AS-PCR method. Similarly, previous studies also reported the incongruence results in both sequencing and AS-PCR methods (Abdalla *et al*., 2008; Sarkar *et al*., 2009b).

As for insensitive acetylcholinesterase, the mutation involved in carbamate and organophosphate resistance which was caused by the replacement of a glycine (GGC) by a serine (AGC) at position 119 of acetylcholinesterase gene have been documented in *Cx. pipiens* Linnaeus, *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus*, *Anopheles nigerrimus*, *An. atroparvus* and *An. sacharovi* since 1980s (Hemingway *et al*., 2004). More recently, this mutation has also been detected in *An. gambiae* and *An. albimanus* (Hemingway *et al*., 2004; Weill *et al*., 2004) while a lack of evidence of this mutation occurs in *Aedes* mosquitoes.

With respect to *Cx. quinquefasciatus*, the distribution of G119S mutation at varying frequencies has been reported (Cui *et al*., 2006; Djogbenou *et al*., 2008). In the current study, a very low frequency of G119S mutation was detected from seven populations, which suggests that the distribution of *ace-1*^{R} is a very recent event in

Malaysia. A similar recent emergence of insensitive acetylcholinesterase has also been described in China (Cui *et al*., 2006).

When comparing the relationship between the frequency of *ace-1*^{R} allele and the status of WHO insecticide susceptibility bioassays in both larval and adult stages, no correlation was found in both stages with regard to propoxur, indicating that other detoxification mechanisms might be involved in these populations. On the other hand, in the larval stage, there was a significant correlation between the malathion resistance ratio and frequency of *ace-1*^{R} allele. Moreover, in adult stage, a significant correlation was also detected between the frequency of *ace-1*^{R} allele and malathion survivability rate. These results suggest that G119S mutation is associated with malathion resistance in *Cx. quinquefasciatus* populations. However, other factors, such as the combination of several detoxification mechanisms could be involved, as reported in Chapter 6.

The findings of this study indicated that RS genotype of 1014F mutation was the most predominant genotype owing to its dispersal across majority of the study sites, while a very low frequency of RR genotype was detected in two populations from Perak and Selangor. As for G119S mutation, SS genotype was the most predominant genotype, while low frequency of RS genotype was detected. The absence of RR genotype of G119S mutation in this study concurred with the findings of Alou *et al*. (2010) where the RR genotype was not detected in carbamate and organophosphate resistance in *An. gambiae* populations from West Africa. Besides, a very low frequency of RR genotype (one out of 100) has also been reported in field populations of *Cx. quinquefasciatus* from West Africa (Djogbénou *et al*., 2008).

Previous studies suggested that the absence of RR genotype in a population might be due to the fitness cost of mutation (Berticat *et al*., 2004; Weill *et al*., 2004; Djogbenou *et al*., 2008), which involve alteration of metabolic and developmental processes that might reduce the fitness-enhancing traits (Davies *et al*., 1996). Indeed,

the insensitivity of insecticide could reduce the normal function of enzyme in resistant individuals (Bourguet *et al*., 1997; Weill *et al*., 2004). As a consequence of high fitness cost, the frequency of RR genotype could decline rapidly after a few generations in the absence of insecticide exposure (Labbe *et al*., 2007). In the present study, it was observed that there was an excess of RS genotype of 1014F mutation recorded in five populations (i.e., Perlis, Penang, Kuala Lumpur, Negeri Sembilan and Malacca), while an excess of RS genotype of G119S mutation recorded in *Cx. quinquefasciatus* population from Sabah. The occurrence of this incidence probably is due to the elimination of RR genotype in fitness cost evolution.

The present study has demonstrated the first appearance of the distribution of L1014F and G119S alleles in Malaysian *Cx. quinquefasciatus* and provided the first field-evolved instance of knockdown resistance and insensitive acetylcholinesterase in insect species in Malaysia. This alarming case in the history of insecticide resistance development would pose a great challenge to both local authorities and researchers in the advancement of vector control management.

CHAPTER 8

GENERAL DISCUSSION

To date, the distribution of mosquito larvae in relation to various habitat characteristics has not yet been fully established in the Southeast Asia region. There is a serious lack of information concerning the breeding preferences of mosquitoes at different locations in this region, including Malaysia. The present study has documented baseline information on the habitat characteristics of *Culex* mosquitoes for the first time at different residential areas in Malaysia. Several correlations (i.e., pH, conductivity, salinity, total dissolved solids, elevation and dissolved oxygen) were found to be associated with Malaysian *Culex* larvae distribution. Previous studies elsewhere have reported a variable relationship between larval density and habitat characteristics (Amerasinghe *et al*., 1995; Minakawa *et al*., 1999; Grillet, 2000; Muturi *et al*., 2008; De Little *et al*., 2009; Jacob *et al*., 2010). Some of these published studies have revealed a lack of significant association between the occurrence of mosquito larvae and habitat characteristics. Regarding this context, an important issue has been addressed by Minakawa *et al*. (1999) where the larval density may be influenced by other habitat characteristics with each contributing some effects or it may be that certain crucial factors have not yet been conclusively identified throughout the study.

A couple of limitations of the present work need to be acknowledged and addressed. Because this surveillance was a nationwide population-based study, it is acknowledged that the weather variables (i.e., seasonal variations of temperature and rainfall) that might affect the distribution of mosquitoes have not been elucidated. Others habitat characteristics, particularly biotic factors such as the presence of

predation, coverage of vegetation and microorganism identification also have not been examined in the current study. It is not clear how other factors affect the female ovipositional behavior. It is possible that these factors may correlate with other habitat characteristics that influence the larval density. Besides, highest level of salinity was recorded from residential area in Terengganu, which is surrounded by the sea and periodically receives inflow of seawater, suggesting that salinity tolerance of mosquito larvae occurred in this area. This interesting finding deserves additional research attention for the investigation of salinity tolerance of mosquito larvae under laboratory and field conditions.

The importance of mosquito-borne diseases can be aggravated when there is an occurrence of mixed infestation between the mosquitoes in a habitat. It could be a serious problem in the attempt to assess their roles as vector-borne diseases during the outbreak of disease transmission. Moreover, over-reliance of insecticide often causes resistant strain to evolve and different species of mosquitoes might have different rates of resistance development towards various classes of insecticides (Hidayati *et al*., 2005). The present study has provided the first documented data on the co-occurrence of mosquito larvae among *Culex* sp., *Lutzia* sp. and *Armigeres* sp. in the residential areas in Malaysia. Nevertheless, the present data is insufficient to interpret the occurrence of mixed infestation caused by interspecific competition, temporal and spatial variation, rapid and extensive urbanization, difference in fecundity between species as well as difference in life cycle duration between species, as pointed out in the previous studies (Chan *et al*., 1971; Reiskind & Wilson, 2008; Leisnham & Juliano, 2009). The current findings indicate that preventive and control measures should be considered proactive when there is an occurrence of mixed infestation between the mosquito species. The preventive and control measures would certainly help to prevent outbreaks of disease

transmission that might be spread by different species of mosquitoes or it'll be too late to instill remedial action when the outbreaks happen.

The findings obtained from this nationwide survey will be a timely reminder to local authorities that effective control measures should be monitored regularly in order to reduce the nuisance of these mosquitoes and the risks of diseases transmission. As noted by WHO (2006), the detailed knowledge of the biology of the target species is of paramount importance in vector control programs because the measures that are effective against one species might be inapplicable for another. Indeed, a description of their distribution patterns and breeding preferences according to habitat characteristics provides useful baseline data for local authorities to justify the application of insecticides in accordance with mosquito species and density. Hence, a more comprehensive study is needed and routine monitoring of vector-borne diseases is indispensable in assisting local authorities to improve vector control strategies currently practiced in Malaysia.

Literature suggested that *Cx. quinquefasciatus* is native to Africa (Vinogradova, 2000). Subsequently, it has been broadly spread throughout tropical, subtropical and warm temperate regions (Vatandoost *et al*., 2004). Notably, this study indicated that *Culex quinquefasciatus* was the most widespread species and well-distributed in urban, suburban, rural as well as remote areas in Malaysia. Apart from Malaysia, its wide range of distribution has also been documented in Thailand (Kitvatanachai *et al*., 2005), Florida (Kline *et al*., 2006), Georgia (Calhoun *et al*., 2007), Argentina (Gleiser & Zalazar, 2010) and India (Kaliwal *et al*., 2010). The broad distribution of this species has gained research interest in regard to its population genetic structure. A worldwide population genetic study of *Cx. quinquefasciatus* comprising the continent of Asia, Africa, South America, North America, Europe and Australia has reported a high level of genetic diversity in Asian and East African populations. However, in this genetic

survey, the *Cx. quinquefasciatus* from Indonesia has only been appointed as the sole representative from Southeast Asia and in fact the genetic background of Southeast Asian *Cx. quinquefasciatus* was overestimated, as evidenced in this study.

Despite the fact that *Cx. quinquefasciatus* was the predominant species in Malaysia, a relatively low level of genetic variability was observed in Malaysian *Cx. quinquefasciatus* populations. Low level of genetic variability observed in the current study contrasts sharply with the previous findings where high level of genetic diversity was found in Asian and East African *Cx. quinquefasciatus* (Fonseca *et al*., 2006). As discussed earlier, low genetic divergence observed in Malaysian populations might be due to the factor of bottleneck effect (Nei *et al*., 1975), *Wolbachia* infection (Rasgon *et al*., 2006; Behbahani, 2012) as well as the evolution of insecticide resistance associated with hitchhiking effect (Yan *et al*., 1998). Among these factors, the issue of insecticide resistance is becoming a matter of great concern in this study. The Malaysian *Cx. quinquefasciatus* that were collected concurrently from the same localities have developed a wide spectrum of insecticide resistance towards DDT, propoxur, malathion and permethrin. It is possible that the fixation of advantageous mutations associated with hitchhiking effect could have occurred in other genome regions around a putative insecticide resistance locus and consequently decreasing the genetic variation (Yan *et al*., 1998).

As proposed previously, it is important to incorporate additional markers that targeted nuclear loci to further confirm the population genetic structure of Malaysian *Cx. quinquefasciatus* because the bottleneck effect on genetic variation is more accentuated in mitochondrial (Birungi & Munstermann, 2002). In addition, the genetic variation of loci surrounding an insecticide resistance locus would be reduced if hitchhiking effect has occurred (Yan *et al*., 1998). There is now an urgent need to investigate the hitchhiking effect associated with insecticide resistance in this mosquito species and

ultimately establish complementary control strategies against these mosquito populations.

Wolbachia, a group of obligate intracellular maternally inherited bacteria that have been commonly found in insects (> 65% of insect species harbor *Wolbachia*) (Brelsfoard & Dobson, 2009). Undoubtedly, the role of *Wolbachia* infection in mosquito populations should not be neglected as previous studies have proven that the *Wolbachia* infected *Cx. quinquefasciatus* populations demonstrated a drastic reduction of mitochondrial variation (Rasgon *et al*., 2006; Behbahani, 2012). Given that the infection of *Wolbachia* is commonly found in Asian *Cx. quinquefasciatus* populations (Kittayapong *et al*., 2000; Ravikuma *et al*., 2011), it is suggested that low mitochondrial diversity observed in the present study may be also attributed by the infection of *Wolbachia*. In recent years, *Wolbachia*-based strategies have been discovered to control insect pests and disease vectors. *Wolbachia*-induced cytoplasmic incompatibility could be used for (1) population suppression, analogous to the sterile insect technique and (2) population replacement, using *Wolbachia* as a vehicle to drive desirable phenotypes into natural populations (Brelsfoard & Dobson, 2009).

As for insecticide susceptibility studies, the conventional WHO adult and larval bioassays revealed the evolution of insecticide resistance against DDT, propoxur, malathion and permethrin in Malaysian *Cx. quinquefasciatus*. The results indicated that both larval and adult bioassays exhibited dissimilar trends in susceptibility, probably due to the differences between the insecticide resistance gene expression in larval and adult stages. Previous studies have indicated that insecticide resistance is more accentuated in the larval stage (Nazni *et al*., 2005; Selvi *et al*., 2006; 2007; Li & Liu, 2010) while a lack of expression was observed in the adult stage (Huchard *et al*., 2006). However, higher levels of insecticide resistance in the adult stage also have been documented (Chavasse & Yap, 1997). In truth, there is still much interesting work to be

done. In this context, the quantification of insecticide resistance gene involving mRNA at different developmental stages of the mosquito could be carried out to provide additional information on the expression of resistance levels towards different insecticides.

Globally, the evolution of multiple or cross insecticide resistance in medically and agriculturally important insect pests is a major limiting factor in the advancement of vector/pest control management (WHO, 2006; Whalon *et al*., 2008). In this respect, statistical analysis indicated that there was a significant correlation between propoxur and permethrin resistance and between propoxur and malathion resistance, suggesting the presence of cross-resistance between different insecticide classes. Previous studies have evidenced that an elevated level of mixed function oxidases conferred cross-resistance between pyrethroids and carbamates (Brooke *et al*., 2000; Cuamba *et al*., 2010). A quick perusal of the present results indicated that most of the populations have revealed elevated enzyme activities of α-esterases, β-esterases, mixed function oxidases, glutathione-S-transferase and acetylcholinesterase, indicating the involvement of multiple resistance mechanisms in these populations and therefore the actual mechanisms that conferred cross-resistance between propoxur and permethrin in this study has not yet been conclusively identified. Meanwhile, cross-resistance between organophosphates and carbamates due to insensitive acetylcholinesterase has been the common phenomenon observed in *Cx. quinquefasciatus* (Cui *et al*., 2006; Alout *et al*., 2007). Comparatively, insensitive acetylcholinesterase against propoxur and malathion inhibitions were also evidenced in this study through the application of both biochemical and molecular assays.

Inversely, cross-resistance within the same class of insecticides has not yet been examined in the present study. Hence, the susceptibility status of this species against other insecticides, particularly the newer pyrethroids (i.e., alpha-cypermethrin, beta-

cyfluthrin, deltamethrin, etofenprox and lambda-cyhalothrin) deserves additional research efforts. Synergist assay is another matter of concern which has not been performed in the current study. The synergist, chemical that inhibits the enzyme could be used to suppress metabolic-based resistance (i.e., triphenylphosphate vs esterases, piperonyl butoxide vs oxidases and ethacrinic acid vs glutathione-S-transferase) and determine the specific resistance mechanisms according to its ability to inhibit specific metabolic pathways (Pasay *et al*., 2009). Under this circumstance, insecticide coupled with synergist will cause the resistant mosquitoes return to apparent susceptibility if the inhibited enzyme is responsible for resistance (Brogdon & McAllister, 1998). With respect to the knockdown rates observed in the adult bioassays, certain populations displayed 0% knockdown making it difficult to choose an appropriate application rate for an adulticiding program. Therefore, it is important to utilize another resistance monitoring methods (i.e., topical application and CDC bottle bioassay) incorporated with synergist assay to further confirm the susceptibility status of this mosquito species in Malaysia.

There has been no comprehensive study which concurrently investigates the roles of α-esterases, β-esterases, mixed function oxidases, glutathione-S-transferase and insensitive acetylcholinesterase in resistance to four major insecticide classes. The biochemical resistance mechanisms that have been reported previously in Malaysian *Cx. quinquefasciatus* populations might be underestimated, especially when there is an occurrence of multiple resistance mechanisms within the same population. In this study, elevated enzyme activities of α-esterases, β-esterases, mixed function oxidases, glutathione-S-transferase and acetylcholinesterase in most of the populations were observed and the incidence of insecticide resistance detected by WHO bioassay was confirmed. An association between α-esterases activity and malathion resistance was found, suggesting that the development of malathion resistance was due to an elevated

level of α-esterases. The involvement of target site alteration in resistance development could not be disregarded, as insensitive acetylcholinesterase associated with malathion resistance in *Cx. quinquefasciatus* was also evidenced by molecular assay in this study.

Besides, an association between activity of α-esterases and β-esterases and between glutathione-S-transferase and acetylcholinesterase was also demonstrated. The actual factors that caused these incidences in Malaysian strains remain questionable and therefore highlight the need for further research. In these circumstances, *in vitro* inhibition test as well as the synergist assay will greatly facilitate the identification of underlying resistance mechanisms. Nevertheless, attempts to relate the occurrence of these incidences with the previous studies reported elsewhere were attempted. It has been found that the association between activity of α-esterases and β-esterases might be due to the co-amplification of two esterase genes (est$\alpha 2^1$ and est$\beta 2^1$) while the association between glutathione-S-transferase and acetylcholinesterase might be due to the development of carbamate resistance.

Knockdown resistance is not a new phenomenon and is an increasing problem in every part of the world. In fact, knockdown resistance has been the subject of research interest among the researchers for more than 50 years and intensive research efforts have unraveled the underlying mechanisms that conferred knockdown resistance at molecular level (Soderlund & Knipple, 2003). Over the past decades, knockdown resistance have been extensively reported worldwide in a number of insect pests (i.e., mosquitoes, cockroaches, ticks, lice, house flies, horn flies, fruit flies, white flies, aphids, beetles and moths) (Soderlund & Knipple, 2003; Hemingway *et al*., 2004; Liu *et al*., 2006) while no evidence has surfaced so far in Malaysian insect species.

As described earlier, both WHO larval and adult bioassays revealed the development of DDT and permethrin resistance in Malaysian *Cx. quinquefasciatus*. In particular, DDT resistance was expressed most frequently, as 0% knockdown was

recorded from 12 out of 14 of the populations. The present study represents a first attempt to characterize the insensitive voltage gated sodium channel and has successfully documented the first field-evolved instance of knockdown resistance in Malaysian insect species. However, the present data failed to demonstrate any correlation between resistance phenotype and frequencies of *kdr* resistant allele in the field populations of Malaysian *Cx. quinquefasciatus*. Although biochemical microassays revealed that there was an increased level of esterases, oxidases and glutathione-S-transferase, but no association was found with regard to DDT and permethrin resistance. This finding again indicates the occurrence of multiple resistance mechanisms which involve both target site alterations and detoxification activities in these populations.

Different resistance levels against propoxur and malathion were detected in Malaysian *Cx. quinquefasciatus* populations. Likewise, the first attempt to characterize the insensitive acetylcholinesterase at molecular level has discovered the recent emergence of G119S mutation in Malaysian populations. Since the frequency of G119S mutation was very low, it is possible that other detoxification mechanisms could be involved in insecticide resistance in this species, as reported by the local researchers in Malaysia (Lee 1990; Selvi *et al*., 2007). It is true that the distribution of G119S mutation was detected at a very low frequency in the present study. Nevertheless, the results demonstrated that malathion resistance was associated with the evolution of G119S mutation and indicated a recent emergence of insensitive acetylcholinesterase in Malaysian *Cx. quinquefasciatus* populations. This first appearance of G119S mutation in Malaysian mosquito is indeed a major problem for both local authorities and researchers to monitor the susceptibility status in the fields. It would pose a great challenge in the management of insecticide resistance and the importance of mosquito-borne diseases can be aggravated when a large proportion of RS genotype present in the

wild populations exhibit the susceptible phenotype. On the other hand, biochemical detection of insensitive acetylcholinesterase revealed a significant increase in acetylcholinesterase activity after propoxur inhibition, indicating that insensitive acetylcholinesterase also played an important role in propoxur resistance.

There have been many arguments about the accuracy of both PCR and sequencing methods for the detection of heterozygous in an individual sample (Simsek *et al*., 2001). Thus, utilization of at least two detection methods or more has been the universal solution. In the present study, the genotyping of insensitive acetylcholinesterase alleles using both DNA sequencing and PCR-RFLP yielded consistent results. As for knockdown resistance detection, it was found that the DNA sequencing results were not in agreement with AS-PCR method. Similarly, previous studies also reported the incongruence results in both sequencing and AS-PCR methods (Abdalla *et al*., 2008; Sarkar *et al*., 2009b). Indeed, the limitations (i.e., high failure rate in the amplification and low precision rate) of the previous AS-PCR methods developed by Martinez-Torres *et al*. (1999) and Chen *et al*. (2000) have been acknowledged in the literatures. In the present study, the latest AS-PCR method developed/modified by Wang *et al*. (2012) was adopted. The authors have confirmed that this newly modified method is both sensitive and specific which could be performed excellently by producing consistent results with *kdr* sequence. However, the present study indicated that this method was not satisfactory for heterozygous determination. Incongruence results obtained from this study bring attention to the need for a more sensitive and efficacy tool in detecting heterozygous mutation at polymorphic sites.

In fact, multiple insecticide resistance involving both metabolic mechanisms and target site alteration has been reported from many parts of the world (Corbel *et al*., 2007; Sarkar *et al*., 2009a; 2009b). In addition to the alteration in target sites and metabolic mechanisms, behavioral resistance has been described. Changes in house entry/exit rate

and feeding time of mosquitoes in reducing the rate of insecticide contact in indoor environment have been observed (Mboho *et al*., 1996; Mathenge *et al*., 2001). On the other hand, reduced insecticide penetration has been noted previously where the resistant insect cuticle has developed a barrier which causes the slow absorption of the chemicals into body (Stone & Brown, 1969; Wood *et al*., 2010; Karaagac, 2012). However, little attention has been given with respect to these mechanisms and the phenomenon is still not fully understood at biochemical level (Hollingworth & Dong, 2008). For future study, a comprehensive investigation of the insecticide resistance involving the factors mentioned above in Malaysian mosquitoes will be beneficial in unraveling the prevailing resistance mechanisms.

Apart from the ascertainment of insecticide resistance mechanisms, several preventive measures should be taken into the consideration in order to reduce the selection pressure that lead to the evolution of insecticide resistance. It has been documented that low insecticide rates may accelerate the evolution of resistance by increasing mutation frequencies (Gressel, 2011), therefore an appropriate dose of insecticide is important for a successful pest control. Application of insecticide rotation by selecting products from different insecticide classes should be carried out to prevent or to reduce the risk of resistance. Besides insecticide application, non-chemical control technique (i.e., biological control) should also be practiced. Biological control of vectors is defined as "the use of predators, parasites and pathogens for the control of vectors" (Lee, 2000). Among the biological control agents, the microbial agents (i.e., *Bacillus thuringiensis israelensis* and *B. sphaericus*) have been widely used in the control of mosquitoes in many parts of the world but not Malaysia. Microbial agents could be served as an alternative to conventional insecticides and it should be incorporated in vector control programs in Malaysia, as recommended by local researchers (Lee, 2000).

In short, application of both biochemical and molecular tools provides significant insights into the evolution and adaptation of Malaysian *Cx. quinquefasciatus*. Nevertheless, various scopes of works still have not been covered in the present study and indeed there is still much work to be done in contributing to the technical know-how of implementing effective vector control programs in Malaysia.

CHAPTER 9

CONCLUSION

1. *Culex quinquefasciatus* was the dominant species in stagnant water in residential areas in Malaysia. *Culex quinquefasciatus* was most likely to exist in the four types of residential areas and *Cx. vishnui* was mainly found in the suburban, rural and remote areas, whereas *Cx. gelidus* was only found in rural areas.

2. Mean number of *Culex* larvae was positively correlated with pH, conductivity, salinity and total dissolved solids. In contrast, the elevation and dissolved oxygen were found negatively correlated with mean number of *Culex* larvae.

3. *Culex quinquefasciatus* was able to breed simultaneously with *Cx. gelidus*, *Lu. fuscanus*, *Cx. vishnui* and *Ar. subalbatus*. On the other hand, *Cx. vishnui* was able to breed simultaneously with *Cx. gelidus* and *Lu. fuscanus*.

4. Genetic diversity of Malaysian *Cx. quinquefasciatus* was extremely low since only three haplotypes (A1-A3) and four haplotypes (B1-B4) were revealed by COI and COII, respectively. The concatenated sequences of COI and COII revealed seven haplotypes (AB1-AB7).

5. It was proposed that haplotype AB1 is the common ancestor of *Cx. quinquefasciatus* and evolved over time into the various haplotypes, namely, AB2, AB3, AB4, AB5, AB6 and AB7, in order to compete with environmental changes and consequently distributed across all states in Malaysia.

6. Malaysian *Cx. quinquefasciatus* shared the same genetic lineage with the East African and Asian *Cx. quinquefasciatus*.

7. Inconsistency of mosquito susceptibility in both larval and adult stages from other districts against other insecticides was found.

8. Overall, the susceptibility status of *Cx. quinquefasciatus* larvae in descending order was: malathion > DDT > propoxur > permethrin, whereas the susceptibility status of *Cx. quinquefasciatus* adults in descending order was: DDT > propoxur > malathion > permethrin.

9. A correlation between propoxur and malathion resistance and between propoxur and permethrin resistance in larval bioassays was found.

10. Elevated enzyme activities of α-esterases, β-esterases, mixed function oxidases, glutathione-S-transferase and acetylcholinesterase were expressed frequently in majority of the populations.

11. An association between α-esterases activity and malathion resistance was found.

12. An association between activity of α-esterases and β-esterases and between glutathione-S-transferase and acetylcholinesterase was demonstrated.

13. This study revealed the first field-evolved instance of L1014F mutation in *Cx. quinquefasciatus*, while the L1014S mutation was not detected across all study sites in Malaysia. RS genotype was detected across all study sites and was most predominant with 99 individuals (out of 140). Perak and Selangor populations indicated the presence of RR genotype but at very low frequencies (3 individuals).

14. This study demonstrated the first field-evolved instance of G119S mutation in Malaysian *Cx. quinquefasciatus*. RS genotype of G119S mutation was detected at a very low frequency (18 out of 140 individuals) while no RR genotype was observed across all study sites in Malaysia.

15. Correlation between the malathion resistance phenotype (in both larval and adult bioassays) and frequency of *ace-1*^{R} allele was demonstrated.

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PRESENTATIONS

Oral Presentations

1. **Low VL**, Chen CD, Lee HL, Leong CS, Chia KHM & Sofian-Azirun M (2011) Insecticide susceptibility status of *Culex quinquefasciatus* larvae obtained from Perak, Terengganu and Kelantan, Malaysia. Universiti Malaysia Terengganu 10th International Annual Symposium, 11-13 July, 2011, Permai Hotel, Kuala Terengganu, Terengganu, Malaysia.

2. **Low VL**, Chen CD, Lee HL, Lim PE, Leong CS & Sofian-Azirun M (2012) Current susceptibility status of Malaysian *Culex quinquefasciatus* Say (Diptera: Culicidae) against four classes of insecticides. The 64th Annual Meeting of the Japan Society of Medical Entomology and Zoology, 29-31 March 2012, Ueda, Japan.

3. **Low VL**, Chen CD, Lim PE, Lee HL & Sofian-Azirun M (2012) Nationwide distribution and insecticide resistance study of Malaysian mosquitoes *Culex quinquefasciatus* Say by molecular and biochemical tools. Seminar on Zoological & Ecological Research in Progress, 18 December 2012, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.

4. **Low VL**, Chen CD, Lim PE, Lee HL, Tan TK, Lim YAL & Sofian-Azirun M (2013) First molecular characterization of insecticide resistance mechanisms in Malaysian *Culex quinquefasciatus*. 49th Annual Scientific Conference of the Malaysian Society of Parasitology and Tropical Medicine, 19-20 March 2013, Grand Seasons Hotel, Jalan Pahang, Kuala Lumpur, Malaysia.

Poster Presentations

1. **Low VL**, Chen CD**,** Lee HL**,** Leong CS & Sofian-Azirun M (2011) Larvicide susceptibility status of *Culex quinquefasciatus* Say obtained from Klang Valley and East Coast, Peninsular Malaysia. 7th Malaysia Indonesia Brunei Medical Sciences Conference, 22-24 July, 2011, Equatorial Hotel, Bangi, Selangor, Malaysia.

2. **Low VL**, Chen CD**,** Lee HL, Leong CS & Sofian-Azirun M (2011) Adulticide susceptibility of *Culex quinquefasciatus* Say populations from Perak, Terengganu and Kelantan, Malaysia 2nd International Symposium on Zoonoses and Emerging Infectious Diseases, 15-16 December 2011, CAIS Auditorium, Universiti Malaysia Sarawak, Malaysia. ***Best Poster Presenter (First Prize)**

3. **Low VL**, Chen CD, Lee HL, Lim PE, Leong CS & Sofian-Azirun M (2012) Mixed breeding of mosquito larvae in stagnant drainage water in residential areas in Malaysia. The 5th Asean Congres of Tropical Medicine and Parasitology, 15-17 May 2012, Manila, Philippines.

4. **Low VL**, Lim PE, Chen CD, Lim YAL, Lee HL, Tan TK & Sofian-Azirun M (2012) Genetic diversity of *Culex quinquefasciatus* Say (Diptera: Culicidae) from Malaysia based on mitochondrial COII sequences. 44th Asia Pacific Academic Consortium for Public Health (APACPH) Conference, 14-17 October 2012, Bandaranaike Memorial International Conference Hall, Colombo, Sri Lanka.

5. **Low VL**, Lim PE, Chen CD, Lim YAL, Lee HL, Tan TK & Sofian-Azirun M (2012) Population genetic structure of Malaysian *Culex quinquefasciatus* Say (Diptera: Culicidae) using mitochondrial COI gene. Australian Entomological Society 43rd AGM & Scientific Conference, 25-28 November 2012, The Old Woolstore, Hobart, Tasmania, Australia.

6. **Low VL**, Chen CD, Lee HL, Tan TK, Chen CF, Leong CS, Lim YAL, Lim PE, Norma-Rashid Y & Sofian-Azirun M. (2013) Biochemical detection of propoxur resistance in *Culex quinquefasciatus* Say populations in Malaysia. 8th Malaysia Indonesia Brunei Medical Sciences Conference, 27-30 June 2013, Universitas Indonesia, Indonesia. ***Best Poster Presenter (Second Prize)**

7. **Low VL**, Chen CD, Lim PE, Lee HL, Lim YAL, Tan TK & Sofian-Azirun M (2013) First molecular genotyping of insensitive acetylcholinesterase in filariasis vector, *Culex quinquefasciatus* Say (Diptera: Culicidae) populations in Malaysia. 24th International Conference of the World Association for the Advancement of Veterinary Parasitology, 25-29 August 2013, Perth Convention Exhibition Centre, Perth, Australia.

PUBLICATIONS

Research Articles

1. **Low VL**, Chen CD, Lee HL, Lim PE, Leong CS & Sofian-Azirun M (2012) Nationwide distribution of *Culex* Mosquitoes and associated habitat characteristics at residential areas in Malaysia. *Journal of the American Mosquito Control Association* 28: 160-169. (**ISI-Cited Publication**)

2. **Low VL**, Chen CD, Lee HL, Lim PE, Leong CS & Sofian-Azirun M (2013) Co-occurrence of mosquito larvae in stagnant water in residential areas in Malaysia. *Asian Biomedicine* 7: 375-380. (**ISI-Cited Publication**)

3. **Low VL**, Lim PE, Chen CD, Lim YAL, Tan TK, Norma-Rashid Y, Lee HL & Sofian-Azirun M (2013) Mitochondrial DNA analyses reveal low genetic diversity in *Culex quinquefasciatus* from residential areas in Malaysia. *Medical and Veterinary Entomology* doi: 10.1111/mve.12022. (**ISI-Cited Publication**)

4. **Low VL**, Chen CD, Lee HL, Lim PE, Leong CS & Sofian-Azirun M (2013) Current susceptibility status of Malaysian *Culex quinquefasciatus* Say (Diptera: Culicidae) against DDT, propoxur, malathion and permethrin. *Journal of Medical Entomology* 50: 103-111. (**ISI-Cited Publication**)

5. **Low VL**, Chen CD, Lim PE, Lee HL, Tan TK, Lim YAL & Sofian-Azirun M (2013) First molecular genotyping of voltage gated sodium channel alleles in *Culex quinquefasciatus* populations in Malaysia. *Pesticide Biochemistry and Physiology* doi: 10.1016/j.pestbp.2013.06.004. (**ISI-Cited Publication**)

6. **Low VL**, Chen CD, Lim PE, Lee HL, Lim YAL, Tan TK & Sofian-Azirun M (2013) First molecular genotyping of insensitive acetylcholinesterase associated with malathion resistance in *Culex quinquefasciatus* Say populations in Malaysia. *Pest Management Science* doi: 10.1002/ps.3512. (**ISI-Cited Publication**)

Proceedings

1. **Low VL**, Chen CD**,** Lee HL**,** Leong CS**,** Chia KHM & Sofian-Azirun M (2011) Insecticide susceptibility status of *Culex quinquefasciatus* larvae obtained from Perak, Terengganu and Kelantan, Malaysia. *Proceeding of Universiti Malaysia Terengganu 10th International Annual Symposium.* pp. 202-204. (ISBN 978-967-5366-60-4).

2. **Low VL**, Chen CD, Lee HL, Leong CS & Sofian-Azirun M (2011) Larvicide susceptibility status of *Culex quinquefasciatus* Say obtained from Klang Valley and East Coast, Peninsular Malaysia. *Medicine and Health* 6 (Supplement): 275-276.

3. **Low VL**, Chen CD, Lee HL, Lim PE, Leong CS & Sofian-Azirun M (2012) Current susceptibility status of Malaysian *Culex quinquefasciatus* Say (Diptera: Culicidae) against four major classes of insecticides. *Medical Entomology and Zoology* 63 (Supplement): 49.

4. **Low VL**, Chen CD, Lee HL, Tan TK, Chen CF, Leong CS, YAL Lim, Lim PE, Norma-Rashid Y & Sofian-Azirun M (2013) Biochemical detection of propoxur resistance in *Culex quinquefasciatus* Say populations in Malaysia. *Proceeding of 8th Malaysia Indonesia Brunei Medical Sciences Conference*. pp. 173. (ISBN 978-979-16208-2-6)

APPENDICES

CTB Type G Bench Protocol

Type G Protocol For Insect / Worm

G-1. Sample Treatment Step

▪ ***Insect / Worm***

■ I. Preparation step ■ II. Disrupt. & Homogen. ■ III. Sample Sizing step □ IV. Pre-Treating step

I. Preparation step

1 Prepare fresh or frozen insect/worm sample

II. Disruption & Homogenization step

2 Slice off the prepared sample to suitable size by blade or scissor

3 Place the sliced sample into mortar

Add liquid nitrogen for sample freezing

Pouring liquid nitrogen slowly, Disrupt and homogenize completely

III. Sample Sizing step

4 Measure 25 mg of sample powder

Transfer to 1.5 ml tube

G-2. DNA Extraction Step

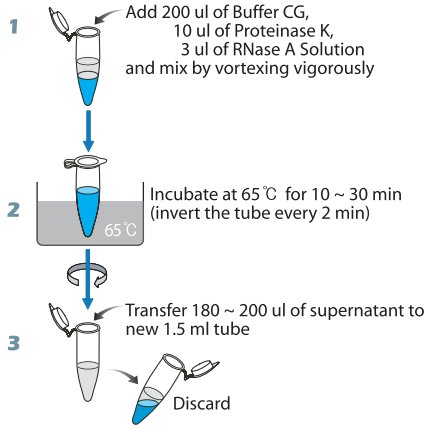
G-2. DNA Extraction Step

▪ ***Insect / Worm***

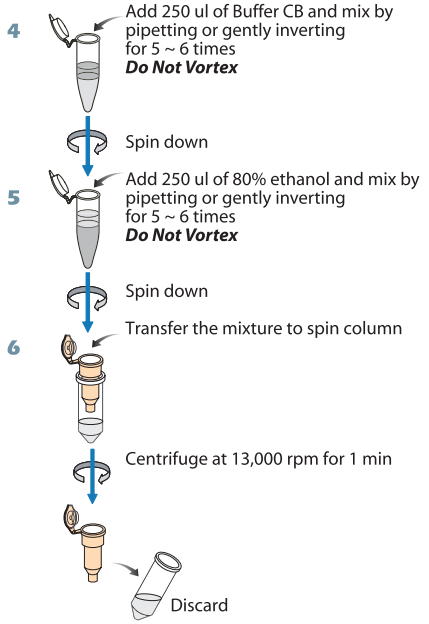
☐ I. Pre-Lysis step ■ II. Lysis step ☐ III. Precipitation step ■ IV. DNA Binding step

☐ V. Washing step A ■ VI. Washing step B ■ VII. Elution step

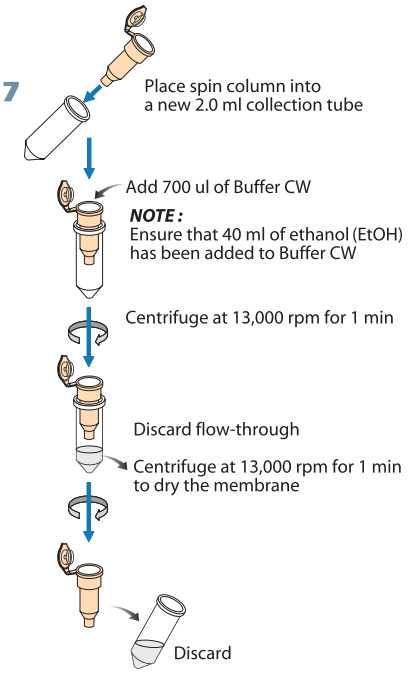
II. Lysis step



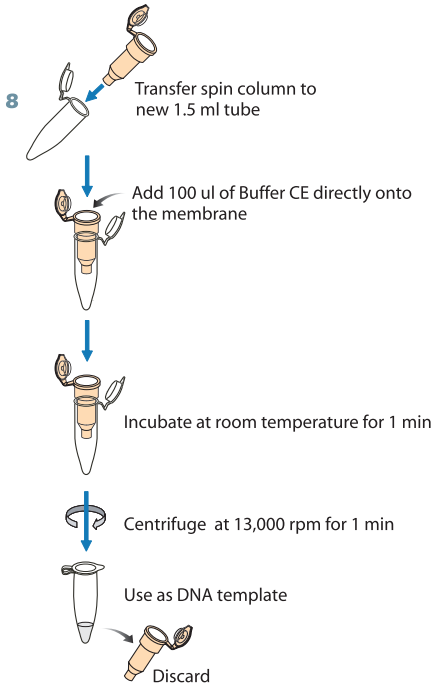
IV. DNA Binding step



VI. Washing step B



VII. Elution step



Type G Protocol
Insect / Worm

G-1. Sample Treatment Step

▪ ***Insect / Worm***

■ I. Preparation step ■ II. Disrupt.& Homogen. ■ III. Sample Sizing step □ IV. Pre-Treating step

This protocol was established for user having a large amount of sample (above 100 mg). In case of a large amount of sample, it is possible to homogenize the sample with mortar and liquid nitrogen. But if you apply few amount of sample, you should be use micropestle and grind the sample directly in a 1.5 ml microcentrifuge tube. Refer to 'Disruption and Homogenization of Important Note' (page 66).

I. Preparation step

1. Prepare fresh or frozen insect / worm sample

The fresh insect or worm sample can be used directly for isolation of genomic DNA. But if the tissues are not used immediately, those should be stored with liquid nitrogen (below -196°C) or deep freezer (below –80°C) for long-term

II. Disruption & Homogenization step

2. Slice off the prepared sample to suitable size by the blade or scissor.

To reduce disruption and homogenization time, we recommend to slice it off.

3. Place the sample material into a grinding jar (mortar). Add liquid nitrogen to the mortar and freeze the sample. Keep the sample submerged in liquid nitrogen, and disrupt carefully until the sample is homogenized completely. Allow the liquid nitrogen to evaporate, and proceed immediately to step 4.

Disruption and homogenization time depends on the tissue samples. We suggest to be disrupted completely until tissue clumps are no longer visible. Clumps of tissue sample will be difficult to lyse properly and will result in a lower yield of DNA. It's very important to keep the sample frozen in liquid nitrogen during disruption and homogenization step to inhibit low DNA yields and degraded DNA. Be careful to handle liquid nitrogen. Generally, it is a fine powder form after disruption and homogenization.

III. Pre-Treating step

4. Measure 25 mg of ground sample, and then transfer into 1.5 ml tube using a spatula.

In order to prevent from thawing the frozen sample during transfer it, use pre-chilled the spatula and 1.5ml tube (When pre-chill the tube, the lid of tube MUST always be OPENed) with liquid nitrogen. The freezing and thawing repetition of frozen sample will result in the DNA degradation. And more, exceeding the recommended optimal amount of starting material will result in inefficient lysis, resulting in low DNA yield and purity.

G-2. DNA Extraction Step

▪ ***Insect / Worm***

☐ I. Pre-Lysis step ■ II. Lysis step ☐ III. Precipitation step ■ IV. DNA Binding step

☐ V. Washing step A ■ VI. Washing step B ■ VII. Elution step

- Equilibrate samples to room temperature (15 ~ 25°C).
- Heat a water bath or heating block to 65°C for use in step 2.
- All centrifugation steps should be carried out at room temperature.

II. Lysis step

1. Add 200 ㎕ Buffer CG, 10 ㎕ Proteinase K and 3 ㎕ RNase A Solution into sample tube and mix by vortexing vigorously.
Be sure that Proteinase K and RNase A solutions are always kept under freezer (below -10°C).

2. Incubate the lysate at 65°C (preheated a heating block or waterbath) for 15 ~ 30 min.
To help lysis sample, mix the tube by inverting every 2 min during the incubation.
Lysis time varies depending on the type of sample. However i-genomic CTB DNA Extraction Mini Kit provides strong lysis mechanism against tissue sample. In case of insect / worm sample, it is enough to lysis completely for 15 ~ 20 mins, respectively.

3. After lysis completely, centrifuge the sample tube to removed un-lysed tissue particles. Then carefully transfer 180 ~ 200 ㎕ of the supernatant into a new 1.5 ml tube (not provided).
If insoluble tissue clumps remains in homogenated mixture, it will be occurred spin column clogging, sometimes. This step helps sample mixing with buffer during binding step by and large. Also It prevents column clogging from insoluble clumps.

IV. DNA Binding step

4. After lysis completely, add 250 ㎕ Buffer CB to the lysate, and mix by pipetting or gently inverting 5 ~ 6 times. DO NOT vortex. After mixing, spin down to remove drops from inside the lid.
It is essential that the sample and Buffer CB are mixed thoroughly to yield a homogeneous solution. It is possible to apply various method of sample mixing (pipetting or inverting) until not showing 2-phase which is not mixed. But do NOT vortex vigorously, because high speed of vortexing can give occasion to shearing of genomic DNA.

5. **Add 250 ㎕ 80% ethanol to the lysate, and mix by pipetting or gently inverting 5 ~ 6 times. DO NOT vortex. After mixing, spin down to remove drops from inside the lid.**
   It is essential that the sample and 80% ethanol are mixed thoroughly to yield a homogeneous solution. It is possible to apply various method of sample mixing (pipetting or inverting) until not showing 2-phase which is not mixed. But do NOT vortex vigorously, because high speed of vortexing can give occasion to shearing of genomic DNA. Do not use alcohols other than ethanol since this may result in reduced yields.
6. **Carefully pipette the whole mixture from step 5 to the spin column (inserted in a 2 ml collection tube) without wetting the rim, centrifuge at 13,000 rpm for 1 min. Discard the flow-through and collection tube altogether.**

VI. Washing step B

7. **Place the spin column into a new 2.0 ml collection tube (additionally supplied), add 700 ㎕ Buffer CW to the spin column, and centrifuge at 13,000 rpm for 1 min. Discard the flow-through, and again centrifuge for additionally 1 min to dry the membrane. Discard the flow-through and collection tube altogether.**
   It is very important to dry the membrane of the spin column since residual ethanol may inhibit subsequent reactions. Following the centrifugation, remove carefully the spin column from the collection tube without contacting with the flow-through, since this will result in carryover of ethanol.
   NOTE : Ensure that 40 ml of ethanol (EtOH) has been added to Buffer CW.

VII. Elution Step

8. **Place the spin column into a new 1.5 ml collection tube (not supplied), add 100 ㎕ Buffer CE directly onto the membrance. Incubate at room temperature for 1 min, and then centrifuge at 13,000 rpm for 1 min to elute.**
   Elution with 50 ㎕ (instead of 100 ㎕) increases the final DNA concentration, but reduces overall DNA yield conventionally.
   NOTE : A new 1.5 ml tube can be used for the second elution step to prevent dilution of the first eluate.
   Alternatively, the tube can be reused for the second elution step to combine the eluates.

Appendix B

For research purpose only. Not for use in diagnostic procedures for clinical purposes. For IN VITRO USE ONLY.

ISO 9001/14001 Certified Company

MEGAquick-spin™

PCR & Agarose Gel DNA Extraction System

Cat. No.	**17281**	**50 Columns**
Cat. No.	**17282**	**250 Columns**

DESCRIPTION

The MEGAquick-spin™ PCR and Agarose Gel DNA Extraction system is designed to extract and purify DNA fragments of 87 bp ~ 20 kb from normal or low-melt agarose gels in either Tris acetate (TAE) or Tris borate (TBE), or to purify PCR products directly from a PCR amplification and DNA cleanup from other enzymatic reactions. Recovery is achieved up to 95%. PCR products are commonly purified to remove excess nucleotides and primers. The BNL Buffer are optimized for efficient recovery of DNA and removal of contaminants. As an added convenience from gel extraction procedures, the BNL Buffer contains a color indicator that allows that easy monitoring of the solution pH for optimal DNA binding. This spin column-based system of the MEGAquick-spin™ kit, which can bind up to 40 ug of DNA, allows recovery of isolated DNA fragments or PCR products in as little as 20 minutes, depending on the number of samples processed and the protocol used.

APPLICATION

MEGAquick-spin™ PCR and Agarose Gel DNA Extraction system is designed for the efficient isolation of DNA fragments from TAE or TBE agarose gels or direct purification of PCR products. The purified DNA can be used for automated fluorescent DNA sequencing, cloning, restriction enzyme digestion and routinely performed DNA manipulation..

KIT CONTENTS AND STORAGE

Label	Description	Contents 50 Columns	Contents 250 Columns
BNL Buffer	Agarose gel lysis buffer	40 ml	170 ml
Washing Buffer (concentrate) [1]	Washing buffer	10 ml	40 ml X 2ea
Elution Buffer	Elution buffer	20 ml	20 ml
MEGAquick-spin™ column (Blue)	Nucleic acid binding column	50 columns	250 columns
Collection tube	2 ml polypropylene tube	50 tubes	250 tubes

[1] Washing Buffer is supplied as concentrate. Add 40 ml (50 columns) or 160 ml (250 columns) per each bottles of ethanol (96~100%) according to the bottle label before use.

- All buffers are stored at room temperature. The term of validity is marked on the box.

ADDITIONAL REQUIRED EQUIPMENT

- Agarose(iNtRON, 32032); scalpel
- Gel running buffer: TAE buffer or TBE buffer Electrophoresis Sterile
- Absolute ethanol
- Standard tabletop microcentrifuge
- Microcentrifuge tubes, sterile (1.5 ml)
- TE buffer (10 mM Tris-HCl, 0.1 mM EDTA; pH 8.0 - 8.5)

PROTOCOL

A. Dissolving the Gel Slice (Agarose Gel DNA Purification)

1. Load and run the gel using an established protocol. DNA can be extracted from standard or low-melt agarose gels in TAE or TBE buffer.
2. After electrophoresis, cut out the interesting DNA fragment with a sharp scalpel or razor blade. Carefully take as much agarose gel as possible.
   Note : If sliced agarose gel put into BNL buffer, the total volume may be increased. When highly concentrated BNL buffer is diluted, and it results low elution efficiency. Therefore, minimize the size of the gel slice by removing extra agarose.
   Note : The gel slice may be stored at 4℃ or -20℃ for up to one week in a tightly closed tube under nuclease-free conditions before purification.
3. Weigh the gel slice in a 1.5 ml tube. Add 3 volumes of BNL buffer to 1 volume of gel (300 μl per 100 mg of agarose gel).
   Note : Add 300 μl of BNL buffer to each 100 mg of gel. If more than 2% of agarose gel, add 6 volumes of BNL buffer.
4. Vortex the mixture and incubate at 55℃ for 10 minutes or until the gel slice is completely dissolved. To help dissolving gel, vortex every 2 ~ 3 min during the incubation.
   Note : Vortex the tube every few minutes to increase the rate of agarose gel melting. Centrifuge the tube briefly at room temperature to ensure the contents are at the bottom of the tube. Once the agarose gel is melted, the gel will not resolidify at room temperature.
   Note : Completely solubilize agarose. For > 2% agarose gel, increase incubation time. BNL Buffer contains a pH indicator which is yellow at pH ≤ 7.5. Indicator enables easy determination of optimal pH for DNA binding. If the mixture pH is > 8.0, color becomes light violet or dark violet which leads to inefficient DNA recovery.
5. (**Optional**) Add 1 gel volume of isopropanol to dissolved gel solution of the step 5 and mix well by pipetting several times. Do not centrifuge after mixing well.
   Note : For < 200 bp of DNA fragment, add 1 volume of isopropanol to 1 volume of gel, and mix well. If the agarose gel slice is 100 mg, add 100 μl of isopropanol. When adding the isopropanol and mixing well by pipetting, small white pellet and clump should be formed. But never mind, and go to the following step. This step increases the yield of DNA fragment. For DNA fragment > 200 bp, adding isopropanol has no effect on yield.
6. To purify the DNA using a microcentrifuge, proceed to **Section C**.

B. Processing PCR Reactions (PCR Product Purification)

1. Amplify target sample using standard amplification conditions.
2. Add an 5 volume of BNL Buffer to the PCR reaction product, and mix well by vortexing. If the PCR product is 20 μl, add 100 μl of BNL buffer to the PCR tube directly.
   Note : Centrifuge the tube briefly at room temperature to ensure the contents are at the bottom of the tube.
3. (**Optional**) For < 200 bp, Add 1.5 volume of isopropanol to the sample and mix well by pipetting several times. Do not centrifuge after mixing well.
   Note : For < 200 bp, Add 1.5 volume of isopropanol, and mix well. If the PCR product is 20 μl, add 100 μl of BNL Buffer and 150 μl of isopropanol. This step increases the yield of DNA fragment.
4. To purify the DNA using a microcentrifuge, proceed to **Section C**.

C. DNA Purification by Centrifugation

1. Place one MEGAquick-spin™ column (blue color) in a Collection Tube for each dissolved gel slice or PCR reaction product.
2. Transfer the dissolved gel mixture or prepared PCR product to the MEGAquick-spin™ column assembly.
3. To bind DNA, apply the sample to the MEGAquick-spin™ column, and centrifuge for 1 min. Discard the flow-through after centrifuging and place the MEGAquick-spin™ column back in the same 2 ml collection tube.
   Note : The maximum volume of the MEGAquick-spin™ column reservoir is 800 μl. For sample volumes of more than 800 μl, simply load and spin again.
4. Add 700 μl of Washing Buffer to column and centrifuge at 13,000 rpm for 1 min. Discard the flow-through after centrifuging and place the MEGAquick- spin™ column back in the same 2 ml collection tube.
   Note : If the DNA will be used for salt sensitive applications, such as blunt-end ligation and direct sequencing, repeat the step 4 using 500 μl of Washing buffer.
5. Centrifuge for 1 min at 13,000 rpm to dry the spin membrane.
   Note : It is important to dry the spin membrane since residual ethanol may interfere with other reactions.
6. Place the MEGAquick-spin™ column to a clean 1.5 ml microcentrifuge tube (not provided). Apply 30 ~ 100 μl of the Elution Buffer directly to the center of the column without touching the membrane with the pipette tip. Incubate at room temperature for 1 minute. Centrifuge for 1 minute at 13,000 rpm.
7. Discard the MEGAquick-spin™ column and store the microcentrifuge tube containing the eluted DNA at -20℃.
   Note : It is suggested to use at least 20 μl of the Elution Buffer to obtain best result.

TECHNICAL INFORMATION

EXPERIMENTAL INFORMATION

• Complete primer removal after PCR

MEGAquick-spin™ PCR & Agarose Gel DNA Extraction System shows not only reliable DNA recovery but complete primer removal efficacy.

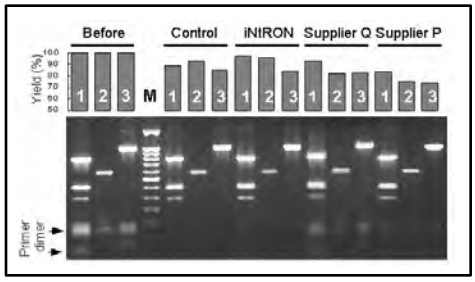


Figure 1. Analysis of PCR product.

The bar-graph shows the recovery of DNA fragment. The values of yield was estimated with TINA 2.0 software.

Before. Before purification; **Control.** PCRquick-spin™ PCR product Purification Kit (iNtRON); **iNtRON.** MEGAquick-spin™ PCR & Agarose Gel DNA Extraction System; **Supplier Q and** P.Q and P company products. **Lane 1.** Multiplex PCR product; **Lane 2.** 570 bp; **Lane 3.** 10 kb; **Lane M.** 100 bp Ladder Molecular Weight DNA Marker.

• Adequate DNA recovery

MEGAquick-spin™ PCR & Agarose Gel Extraction System assures of the suitable recovery of DNA fragment from agarose gel.

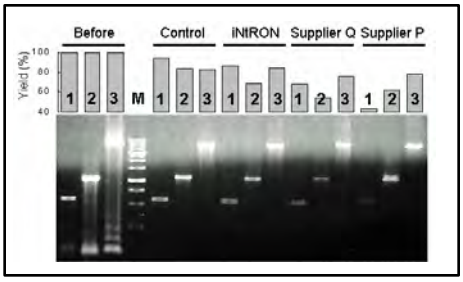


Figure 2. Size exclusion by agarose gel extraction.

The bar-graph shows the recovery of DNA fragment. The value of yield was estimated with TINA 2.0 software.

Before. Before purification; **Control.** MEGA-spin™ Agarose Gel DNA Extraction Kit (iNtRON); **iNtRON.** MEGAquick-spin™ PCR & Agarose Gel DNA Extraction System; **Supplier Q and P.** Q and P company products; **Lane 1.** 570 bp; **Lane 2.** 1.3 kb; **Lane 3.** 4.5 kb; **Lane M.** 1 kb Ladder Molecular Weight DNA Marker.

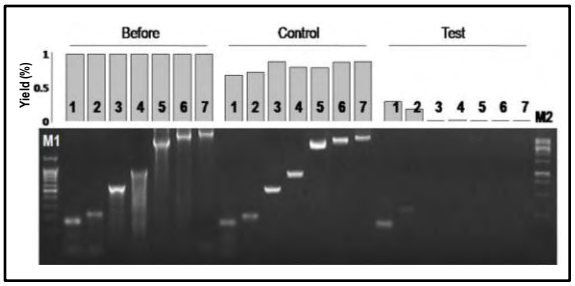


Figure 3. pH dependence of DNA recovery to MEGAquick-spin™ PCR & Agarose Gel DNA Extraction System.

DNA fragments were extracted from gel of different pH condition. Panel Test show that inefficient DNA recovery in pH too high. In contrast to Panel Control show that sufficient DNA recovery in optimal pH within MEGAquick-spin™ PCR & Agarose Gel DNA Extraction System BNL Buffer. The bar-graph shows the recovery of DNA fragment. The value of yield was estimated with TINA 2.0 software.

Before. Before gel extraction; **Control.** MEGAquick-spin™ PCR & Agarose Gel DNA Extraction System; **Test.** BNL Buffer mixture pH too high; **Lane 1.** 161 bp; **Lane 2.** 218 bp; **Lane 3.** 570 bp; **Lane 4.** 1 kb; **Lane 5.** 4.5 kb; **Lane 6.** 9 kb; **Lane 7.** 20 kb; **Lane M1.** 100 bp Ladder Molecular Weight DNA Marker; **Lane M2.** 1 kb Ladder Molecular Weight DNA Marker.

TROUBLESHOOTING GUIDE

Problem	Possible Cause	Recommendation
Low or no DNA yield	Washing buffer did not contain ethanol	- Ethanol must be added to Washing buffer before use.
	Inappropriate elution buffer	- DNA will only be eluted in low salt buffer or water.
	Incorrect volume of BNL buffer	- Verify that a correct volume of BNL buffer was added to the Gel slice.
	Gel slice incompletely solubilized	- After addition of BNL buffer to the slice, mix by vortexing the tube every 2 minutes during the 55 ℃ incubation.
	Cloudy and gelatinous appearance of sample mixture after addition of isopropanol	- This may be due to salt contamination, and will be disappear by mixing the sample. Alternatively, the gel slice may not be completely solubilzed. The concentration of gel may be above 2%. In this case, apply the 6 volume of BNL buffer to gel slice, and melt the gel completely.
DNA does not perform well, e.g., in enzyme reaction, ligation, sequencing reactions	Salt concentration in eluate too high	- Modify the washing step by incubating the column for 5min at RT after adding 700 μl of Washing Buffer and the centrifuge.
	Eluate contaminated with agarose	- If The gel slice is incompletely solubilized or over-weighed, increased the incubation time.
	Eluate contains residual ethanol	- Ensure that the wash flow-through is drained from the collection tube and that the column is then centrifuged at 13,000 rpm for 1min.
	Eluate contain primer-dimers	- Primer dimers formed are longer than 50bp, and are not completely removed. After binding step, wash the column with 750 μl of a 35% guanidine hydrochloride aqueous solution. Follow with the washing, and elution step as in the protocols.
BNL buffer become violet color.	pH of electrophoresis buffer too high	- The electrophoresis buffer has been repeatedly used or incorrectly prepared, resulting in a sample pH that exceeds the buffering capacity of BNL buffer and leads to inefficient DNA binding. Add 0.1 volume of 3M sodium acetate, pH 5.0, to the sample and mix.

RELATED PRODUCTS

Product Name	Cat.No.
PCRquick-spin™ PCR Product Purification Kit	17202
MEGA-spin™ Agarose Gel DNA Extraction Kit	17183
DNA-spin™ Plasmid DNA Purification Kit	17093
Maxime™ PCR PreMix (i-StarTaq)	25165
Maxime™ PCR PreMix (i-pfu)	25185
100 bp Ladder Molecular Weight DNA Marker	24012
1 kb Ladder Molecular Weight DNA Marker	24022

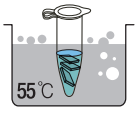
Simple Protocol : Agaros gel

Prepare DNA band from agarose gel

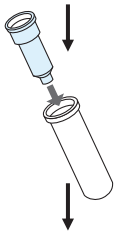
Weigh the sliced agaros gel

Transfer the sliced agarose gel to
1.5 ml tube

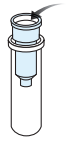
Add **BNL Buffer** as same as
3 times of sliced gel volume.



Vortex and incubate at 50 ~ 55℃
for 10 min.

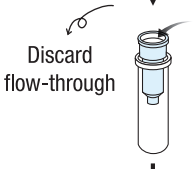


Prepare Spin Column to
Collection tube



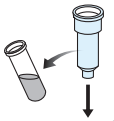
Add Sample to Spin Column

Centrifuge at 13,000 rpm/1 min.

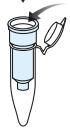


Add 700 ㎕ of **Washing buffer**

Centrifuge at 13,000 rpm/1 min.

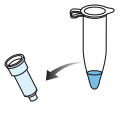


Discard Collection tube



Add 30 ~ 100 ㎕ of **Elution buffer**
and incubate for 1 min. at R.T

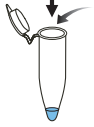
Centrifuge at 13,000 rpm/1 min.



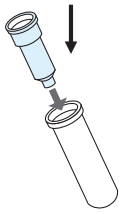
Discard Spin column

Simple Protocol : PCR product

PCR



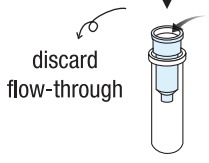
Add **BNL Buffer** as same as
5 times of PCR volume



Prepare Spin Column to
Collection tube

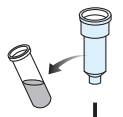
Add Sample to Spin Column

Centrifuge at 13,000 rpm/1 min.

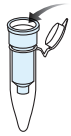


Add 700 ㎕ of **Washing buffer**

Centrifuge at 13,000 rpm/1 min.

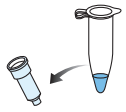


Discard Collection tube



Add 30 ~ 100 ㎕ of **Elution buffer**
and incubate for 1 min. at R.T

Centrifuge at 13,000 rpm/1 min.



Discard Spin column

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NATIONWIDE DISTRIBUTION OF *CULEX* MOSQUITOES AND ASSOCIATED HABITAT CHARACTERISTICS AT RESIDENTIAL AREAS IN MALAYSIA

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ABSTRACT. A standardized larval dipping method was used to determine the infestation rates of *Culex* and other species of mosquitoes in stagnant water at 20 residential areas. This study also examined the associations between *Culex* distribution and various habitat characteristics across all states in Malaysia. Identification of 7,848 specimens yielded 6 species dominated by *Culex quinquefasciatus* (82.74%), followed by *Cx. vishui* (14.39%), *Cx. gelidus* (2.70%), *Lutzia fuscanus* (0.11%), *Armigeres subalbatus* (0.05%), and *Anopheles separatus* (0.01%). The *Culex* larvae occurred in stagnant water with pH ranging from 6.4 to 8.2; conductivity, 139.7 to 6635.2 µs/cm; salinity, 0.07 to 3.64 ppt; total dissolved solids, 0.09 to 4.27g/liter; and dissolved oxygen, 5.11 to 8.11 mg/liter. The mean number of *Culex* larvae was positively correlated with pH, conductivity, salinity, and total dissolved solids. In contrast, the elevation and dissolved oxygen were found negatively correlated with mean number of *Culex* larvae. This study documented baseline information on the habitat characteristics of *Culex* species for the 1st time at different residential areas in Malaysia. The findings of this study will be a timely reminder to local authorities that effective control measures should be monitored regularly in order to reduce the nuisance of these mosquitoes and the risks of disease transmission.

KEY WORDS Nationwide surveillance, *Culex* mosquitoes, habitat characteristics, breeding index, dipper index, Malaysia

INTRODUCTION

The infectious diseases carried by mosquito vectors have been an increasing public health concern in recent decades. The mosquito-borne diseases and their vectors have been well documented in every part of the world including Malaysia. There are 442 species of mosquito representing 20 genera recorded in Malaysia (Miyagi and Toma 2000). Several species of Malaysian mosquitoes have been incriminated as important public health vectors in disease transmission. In this region, *Aedes aegypti* (L.) and *Ae. albopictus* (Skuse) are the dengue vectors (Lee and Inder 1993); *Culex gelidius* (Theobald) is the principal vector of Japanese encephalitis (Vythilingam et al. 1994), whereas *Cx. quinquefasciatus* Say is known for bancroftian filariasis (Vythilingam et al. 2005). *Mansonia uniformis* (Theobald), *Ma. annulifera* (Theobald), *Ma. annulata* (Leicester), *Ma. bonneae* (Edwards), *Ma. dives* (Schiner), and *Ma. indiana* (Edwards) are vectors of brugian filariasis (Wharton 1962) and *Anopheles maculates* (Theobald), *An. balabacensis* (Baisas), *An. dirus* (Peyton and Harrison), *An. letifer* (Sandosham), *An. campestris* (Reid), *An. sundaicus* (Rodenwaldt), *An. donaldi* (Reid), *An. leucosphyrus* (Doenitz), and *An. flavirostris* (Ludlow) are all vectors of malaria (Rahman et al. 1997).

Knowledge of their distribution in different environments needs to be ascertained. Mosquito surveillance remains the preliminary step used in vector monitoring and control. Mosquito surveillance of larval and adult stages by different approaches has been frequently reported in Malaysia. The population studies of mosquito vectors have been carried out by using human landing catches (Reid 1961, Rohani et al. 1999, Tan et al. 2008). In addition to identifying the mosquito breeding sites, container surveys have also been conducted (Cheah et al. 2006, Chen et al. 2009, Nyamah et al. 2010). Moreover, mosquito studies using animal-baited traps have been reported in the literature (Reid 1961, Rohani et al. 1999, Tan et al. 2008). The use of light traps in mosquito population studies have also been documented (Vythilingam et al. 1992, Oli et al. 2005). The use of ovitraps in dengue vector surveillance has been the focus of many studies in recent years (Chen et al. 2005, 2006, 2009; Cheah et al. 2006). Larval dipping is another approach used in larval population studies in the states of Pahang and Penang (Hassan et al. 2010, Rohani et al. 2010). However, little attention is being paid to larval dipping in East Malaysia. The distribution of mosquito larvae in relation to various habitat characteristics has not yet been fully elucidated in the Southeast Asia region. There is a lack of information regarding the breeding preferences at different locations in this region. Apart from Southeast Asia, previous studies

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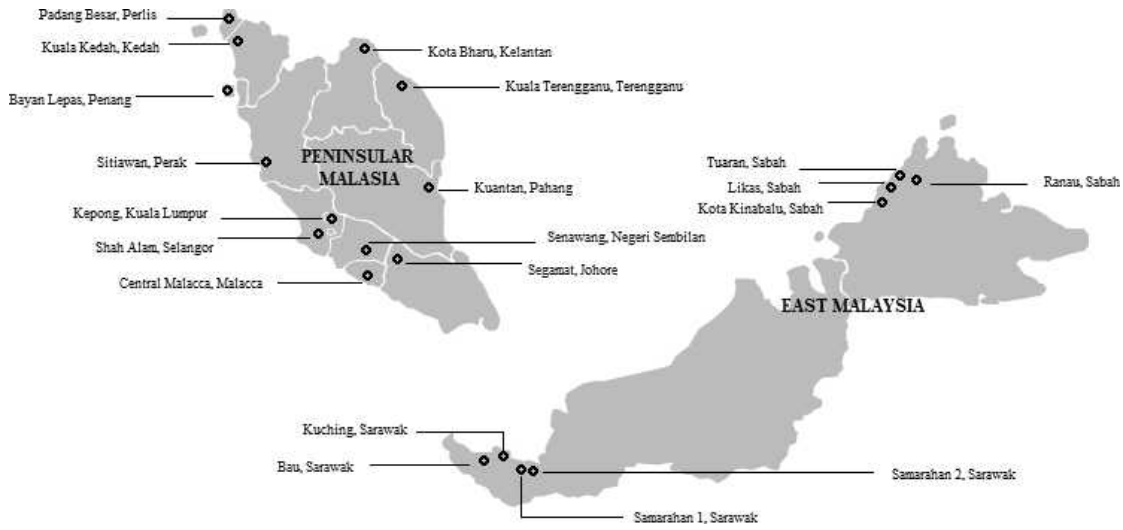

Fig. 1. Location of study sites in Peninsular and East Malaysia.

elsewhere have reported a variable relationship between larval density and habitat characteristics (Amerasinghe et al. 1995, Minakawa et al. 1999, Grillet 2000, Muturi et al. 2008, De Little et al. 2009, Jacob et al. 2010).

To date, a nationwide surveillance of *Culex* larvae that primarily live in stagnant water has not yet been conducted in Malaysia. Hence, the present study attempts to 1) determine the infestation rates of *Culex* and other species of mosquitoes in stagnant water as part of an ongoing entomological investigation and 2) provide the 1st documented data on associations between the *Culex* distribution and various habitat characteristics in residential areas in all states of Peninsular Malaysia and East Malaysia. The findings of this study will be useful for vector control operations in these areas.

MATERIALS AND METHODS

Study areas

The larval surveillance was conducted at 20 residential areas in Peninsular Malaysia and East Malaysia from February to July 2011. The geographical description of the study sites is presented in Fig. 1 and Table 1. There is no distinct wet or dry season throughout the year and rain is experienced every single month. However, seasonal rainfall variation occurred in every part of Malaysia during the northeast and northwest monsoon seasons. It has been confirmed that the sample collection during the study period was free from its influence across all states. The annual rainfall in all sites exceeds 2,000 mm. All study sites have a tropical climate with an average temperature of 32°C and a relative humidity of 80% (Chen et al. 2006).

Larval dipping method

A total of 1,863 typical sources of stagnant water: drains, pools, reservoirs, canals, and temporary flooded areas were surveyed for the presence of mosquito species (targeting *Culex* species) that primarily breed in stagnant water. In order to prevent water quality changes by heavy rain, all samplings were carried out at least 3 days after rain. Mosquito larvae were dipped from stagnant water by using a 330-ml capacity dipper. Since there has always been a problem in relation to total number of dips taken according to the size of breeding sites, a standard dipping technique developed by Mendoza et al. (2008) was carried out in present study. Standardization of the number of dips in accordance with the surface area of the water body was as follows: number of dips, water surface area (m^2): 1, <0.25; 2, 0.26–1.0; 3, 1.1–3.0; 4, 3.1–5.0; 5, 5.1–7.0; 6, 7.1–9.0; and so on. Dips were taken gently with a 2–3-min pause, to allow for the mosquito larvae to move freely in the air–water interface. Water samples were collected from sites where mosquito larvae were present. The pH, conductivity, salinity, total dissolved solids (TDS), and dissolved oxygen (DO) of the water samples were measured by using a handheld water quality meter (YSI® 556 Multi-Probe System, Yellow Springs, OH). The elevation and coordinates of each study site were recorded by using Garmin® GPS 72H (Olathe, KS).

Species identification

Field-collected larvae were placed in 500-ml plastic cups and transported to the laboratory for identification. The larvae were placed in larval rearing trays containing deionized water and

Table 1. Geographical description of study sites.

Malaysia	Region	State	District	Study site[1]	Coordinates	Elevation (m)	Landscape
Peninsular	East Coast	Kelantan	Kota Bharu	Taman Guru	06°05′49.43″N, 102°14′06.80″E	8.53	Suburban
		Terengganu	Kuala Terengganu	Kg. Simpang Empat	05°15′57.73″N, 103°10′49.90″E	6.71	Rural
		Pahang	Kuantan	Taman Chenderawasih	03°48′00.40″N, 103°18′02.20″E	6.40	Suburban
	Northern	Perlis	Padang Besar	Taman Singgahsana	06°39′11.00″N, 100°18′54.00″E	52.43	Rural
		Kedah	Kuala Kedah	Taman Selat	06°05′02.10″N, 100°18′07.70″E	6.71	Suburban
		Penang	Bayan Lepas	Taman Bayan Baru	05°19′46.51″N, 100°17′24.80″E	8.84	Urban
		Perak	Sitiawan	Taman Bunga Ros	04°12′42.21″N, 100°41′42.20″E	9.75	Suburban
	Central	Selangor	Shah Alam	Section 17	03°02′58.28″N, 101°30′16.40″E	5.18	Urban
		Kuala Lumpur	Kepong	Kepong Baru	03°12′18.23″N, 101°38′43.60″E	50.60	Urban
	Southern	Negeri Sembilan	Senawang	Taman Marida	02°41′52.40″N, 101°59′02.44″E	79.86	Suburban
		Malacca	Central Malacca	Kg. Pengkalan Rama Pantai	02°12′35.77″N, 102°15′02.52″E	6.71	Rural
		Johore	Segamat	Segamat Baru	02°29′56.50″N, 102°51′12.10″E	25.60	Suburban
East Malaysia	West	Sarawak	Kuching	RPR Batu Kawa	01°31′20.50″N, 110°19′01.30″E	9.75	Suburban
			Bau	Kg. Skiat Baru	01°23′53.40″N, 110°11′11.70″E	30.18	Remote
			Samarahan 1	Kg. Rembus	01°28′59.90″N, 110°28′59.90″E	5.18	Remote
			Samarahan 2	Kg. Baru	01°29′19.40″N, 110°30′24.40″E	6.71	Remote
	East	Sabah	Tuaran	Taman Kolej Tuaran	06°10′48.90″N, 116°13′41.30″E	18.59	Rural
			Likas	Taman Kingfisher	06°01′22.68″N, 116°07′22.50″E	10.06	Suburban
			Ranau	Taman Delima	06°00′16.92″N, 116°48′34.90″E	444.40	Rural
			Kota Kinabalu	Taman Kepayan	05°56′24.96″N, 116°04′22.20″E	15.24	Suburban

[1] Kg., Kampung.

provided with a fine mixture of mice chow, beef liver, and milk powder in the ratio of 2:1:1 by weight. The pupae were sorted out daily and introduced into a mosquito cage. The emerging adults were killed using ethyl acetate before mounting on points. Moribund and dead larvae were subsequently mounted for identification. The adults and larvae were identified according to illustrated keys (Rattanarithikul et al. 2005, 2006) and cross-referenced with the voucher specimens from the laboratory. Representative specimens from this study were used as voucher specimens and deposited in the Laboratory of Zoological and Ecological Network, University of Malaya.

Statistical analysis

Data were analyzed to determine the following: 1) dipper index (DI), the percentage of positive dips against the total number of dips taken, 2) mean number of larvae per dip, and 3) breeding index (BI), developed by Belkin (1954).

Breeding index was calculated as

$$BI = TLP/ND \times BP$$

where BI = breeding index, TLP = total number of larvae, ND = number of dips, and BP = number of breeding places. The breeding place was defined as each separate microhabitat or station within a site from which 1 to 3 positive dips were obtained.

Data were analyzed using the statistical program, SPSS version 18 (Chicago, IL). Descriptive statistics were used to summarize the data for each study area. The differences between mean number of larvae per dip across all study sites were assessed by 1-way ANOVA. Spearman rank-order correlation was used to determine the associations between mean number of *Culex* larvae and habitat characteristics.

RESULTS

The data presented clearly indicated the study sites as natural breeding sites of mosquitoes, particularly *Culex* spp. A total of 3,117 dips were performed at 20 sampling sites, representing 4 types of residential areas: urban (n = 3), suburban (n = 8), rural (n = 6), and remote (n = 3). A total of 547 positive dips were identified, out of 3,117 dips. A total of 7,848 specimens belonging to 4 genera, namely *Culex*, *Armigeres*, *Anopheles*, and *Lutzia* were collected. *Culex quinquefasciatus* (82.74%) was the dominant species, followed by *Cx. vishnui* (Theobald) (14.39%) and *Cx. gelidus* (2.70%). In addition, *Lu. fuscanus* (Wiedemann) (0.11%), *Ar. subalbatus* (Coquillett) (0.05%), and *An. separatus* (Leicester) (0.01%) were also detected in small numbers (Table 2). The distribution of *Culex* spp. in 4 types of residential areas is represented in

Table 2. Total number and percentage of mosquito larvae collected from various study sites.

Study site	Total no. of dips	*Culex quinquefasciatus*		*Cx. vishnui*		*Cx. gelidus*		*Armigeres subalbatus*		*Lutzia fuscanus*		*Anopheles separatus*		Total no. of larvae
		n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	
Kota Bharu, Kelantan	121	1,204	99.75	0	0.00	0	0.00	0	0.00	3	0.25	0	0.00	1,207
Kuala Terengganu, Terengganu	197	138	19.80	559	80.20	0	0.00	0	0.00	0	0.00	0	0.00	697
Kuantan, Pahang	167	30	100.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	30
Padang Besar, Perlis	123	98	100.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	98
Kuala Kedah, Kedah	144	47	87.04	7	12.96	0	0.00	0	0.00	0	0.00	0	0.00	54
Bayan Lepas, Penang	212	332	100.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	332
Sitiawan, Perak	196	688	100.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	688
Shah Alam, Selangor	187	1,383	99.93	0	0.00	0	0.00	0	0.00	1	0.07	0	0.00	1,384
Kepong, Kuala Lumpur	162	482	100.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	482
Senawang, Negeri Sembilan	331	155	97.48	0	0.00	0	0.00	0	0.00	4	2.52	0	0.00	159
Central Malacca, Malacca	131	445	99.55	0	0.00	0	0.00	2	0.45	0	0.00	0	0.00	447
Segamat, Johore	267	720	99.72	0	0.00	0	0.00	2	0.28	0	0.00	0	0.00	722
Kuching, Sarawak	218	374	79.75	94	20.04	0	0.00	0	0.00	1	0.21	0	0.00	469
Bau, Sarawak	86	0	0.00	7	100.00	0	0.00	0	0.00	0	0.00	0	0.00	7
Samarahan 1, Sarawak	103	6	20.69	23	79.31	0	0.00	0	0.00	0	0.00	0	0.00	29
Samarahan 2, Sarawak	113	8	100.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	8
Tuaran, Sabah	46	19	5.05	150	39.89	207	55.06	0	0.00	0	0.00	0	0.00	376
Likas, Sabah	162	15	4.93	289	95.07	0	0.00	0	0.00	0	0.00	0	0.00	304
Ranau, Sabah	53	4	40.00	0	0.00	5	50.00	0	0.00	0	0.00	1	10.00	10
Kota Kinabalu, Sabah	98	345	100.00	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	345
Total	3117	6,493	82.74	1129	14.39	212	2.70	4	0.05	9	0.11	1	0.01	7,848

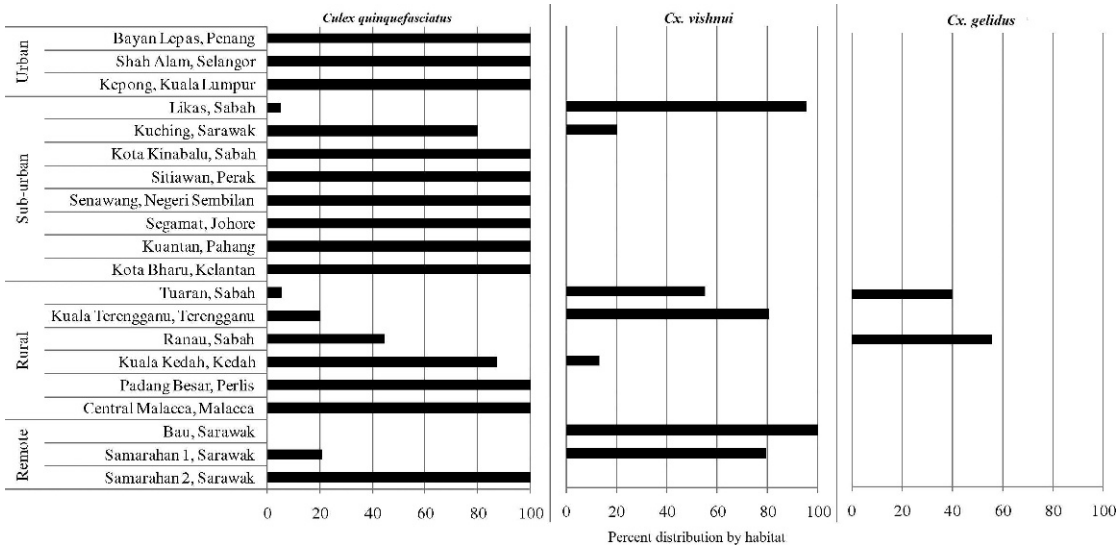

Fig. 2. Distribution of *Culex* larvae in stagnant drainage water in 4 types of residential areas in Malaysia.

Fig. 2. Overall, *Cx. quinquefasciatus* was most likely to exist in the 4 types of residential areas and *Cx. vishnui* was mainly found in the suburban, rural, and remote areas, whereas *Cx. gelidus* was only found in rural areas.

The DI and BI of mosquito larvae are presented in Table 3. The mean number of larvae per dip at from Kota Bharu (Kelantan) was significantly higher across all study sites ($F = 9.73$, df = 3, 116, $P = 0.000$). High DI values were recorded in Kota Bharu (Kelantan), Tuaran (Sabah), Sitiawan (Perak), and Central Malacca (Malacca), accounting for 46.28%, 43.48%, 41.33%, and 40.46%, respectively. The highest BI value, 65.39, was found in Tuaran (Sabah).

The *Culex* larvae occurred in stagnant water with pH ranging from 6.4 to 8.2; conductivity, 139.7 to 6635.2 µs/cm; salinity, 0.07 to 3.64 ppt; TDS, 0.09 to 4.27 g/liter; and DO, 5.11 to 8.11 mg/liter (Table 4). The correlation between the mean number of *Culex* larvae and habitat characteristics are presented in Fig. 3. The Spearman rank-order correlation revealed that the mean number of *Culex* larvae was positively correlated with pH

Table 3. Dipper index, mean number of larvae per dip, and breeding index obtained at various study sites.

Study site	No.	No. of positive dips	Dipper index	Mean no. of larvae per dip[1]	Breeding index
Kota Bharu, Kelantan	121	56	46.28%	9.98 ± 1.59	49.88
Kuala Terengganu, Terengganu	197	59	29.95%	3.54 ± 0.54	17.69
Kuantan, Pahang	167	19	11.38%	0.18 ± 0.05	0.72
Padang Besar, Perlis	123	11	8.94%	0.80 ± 0.28	1.59
Kuala Kedah, Kedah	144	17	11.81%	0.36 ± 0.10	1.88
Bayan Lepas, Penang	212	10	4.72%	1.57 ± 0.64	1.57
Sitiawan, Perak	196	81	41.33%	3.51 ± 0.45	21.06
Shah Alam, Selangor	187	34	18.18%	7.40 ± 1.61	22.20
Kepong, Kuala Lumpur	162	31	19.14%	2.98 ± 0.75	8.93
Senawang, Negeri Sembilan	331	15	4.53%	0.48 ± 0.19	0.48
Central Malacca, Malacca	131	53	40.46%	3.41 ± 0.63	17.06
Segamat, Johore	267	78	29.21%	2.70 ± 0.41	13.50
Kuching, Sarawak	218	30	13.76%	1.72 ± 0.59	15.06
Bau, Sarawak	86	5	5.81%	0.08 ± 0.04	0.08
Samarahan 1, Sarawak	103	10	9.71%	0.28 ± 0.10	1.69
Samarahan 2, Sarawak	113	3	2.65%	0.07 ± 0.04	0.14
Tuaran, Sabah	46	20	43.48%	7.99 ± 3.05	65.39
Likas, Sabah	162	8	4.94%	1.87 ± 0.91	5.63
Ranau, Sabah	53	2	3.77%	0.19 ± 0.17	0.38
Kota Kinabalu, Sabah	98	5	5.10%	3.52 ± 2.44	10.56

[1] $F = 9.73$, df = 3, 116, $P = 0.000$.

Table 4. Water quality data of stagnant water samples from all study sites.

Study site	pH	Conductivity (μs/cm)	Salinity (ppt)	Total dissolved solids (g/liter)	Dissolved oxygen (mg/liter)
Kota Bharu, Kelantan	7.80 ± 0.09	364.57 ± 58.84	0.17 ± 0.03	0.24 ± 0.04	7.29 ± 0.13
Kuala Terengganu, Terengganu	7.82 ± 0.10	6635.17 ± 1391.64	3.64 ± 0.80	4.27 ± 0.90	6.23 ± 0.09
Kuantan, Pahang	6.66 ± 0.12	232.33 ± 9.84	0.11 ± 0.00	0.15 ± 0.01	8.11 ± 0.09
Padang Besar, Perlis	7.56 ± 0.14	1532.00 ± 760.08	0.81 ± 0.41	1.02 ± 0.51	5.11 ± 1.73
Kuala Kedah, Kedah	7.27 ± 0.16	617.00 ± 18.02	0.30 ± 0.01	0.41 ± 0.01	6.22 ± 0.89
Bayan Lepas, Penang	7.17 ± 0.11	277.40 ± 7.83	0.11 ± 0.03	0.15 ± 0.04	7.86 ± 0.05
Sitiawan, Perak	7.50 ± 0.03	362.50 ± 3.23	0.18 ± 0.01	0.24 ± 0.00	5.98 ± 0.23
Shah Alam, Selangor	7.90 ± 0.11	337.00 ± 30.53	0.16 ± 0.02	0.23 ± 0.02	7.90 ± 0.16
Kepong, Kuala Lumpur	7.79 ± 0.07	602.50 ± 62.06	0.30 ± 0.03	0.40 ± 0.04	7.72 ± 0.36
Senawang, Negeri Sembilan	8.22 ± 0.09	569.50 ± 54.48	0.28 ± 0.03	0.37 ± 0.04	7.48 ± 0.22
Central Malacca, Malacca	6.92 ± 0.10	589.00 ± 55.65	0.29 ± 0.03	0.39 ± 0.04	6.42 ± 0.55
Segamat, Johore	6.42 ± 0.34	304.67 ± 22.20	0.15 ± 0.01	0.20 ± 0.02	7.63 ± 0.28
Kuching, Sarawak	7.12 ± 0.06	365.20 ± 66.75	0.18 ± 0.03	0.24 ± 0.04	6.50 ± 0.51
Bau, Sarawak	7.41 ± 0.02	192.00 ± 5.51	0.09 ± 0.00	0.13 ± 0.00	7.57 ± 0.28
Samarahan 1, Sarawak	7.34 ± 0.06	195.00 ± 10.82	0.09 ± 0.01	0.13 ± 0.01	6.52 ± 0.40
Samarahan 2, Sarawak	7.21 ± 0.04	690.50 ± 5.50	0.33 ± 0.01	0.45 ± 0.01	6.81 ± 0.13
Tuaran, Sabah	6.79 ± 0.02	139.67 ± 7.94	0.07 ± 0.00	0.09 ± 0.01	7.75 ± 0.17
Likas, Sabah	6.54 ± 0.09	334.67 ± 79.41	0.16 ± 0.04	0.22 ± 0.05	5.59 ± 0.59
Ranau, Sabah	6.81 ± 0.10	338.00 ± 133.00	0.17 ± 0.07	0.23 ± 0.09	7.47 ± 0.07
Kota Kinabalu, Sabah	6.63 ± 0.22	340.40 ± 32.88	0.17 ± 0.02	0.23 ± 0.02	6.61 ± 0.68

($r = 0.521$, $P = 0.040$), conductivity ($r = 0.574$, $P = 0.022$), salinity ($r = 0.510$, $P = 0.045$), and TDS ($r = 0.591$, $P = 0.017$). These positive correlations implied that the infestation rates of *Culex* larvae increased with increasing pH, conductivity, salinity, and TDS. Conversely, the elevation ($r = -0.657$, $P = 0.005$) and DO ($r = -0.415$, $P = 0.109$) were found to be negatively correlated with the mean number of *Culex* larvae. These negative correlations implied that the infestation rates of *Culex* larvae decreased with increasing elevation and DO.

DISCUSSION

Based on the entomological surveys conducted in the current study, *Culex* mosquitoes appeared to be the most abundant species found across all study sites, hence confirming that stagnant water from the residential areas provided suitable larval sites for *Culex* mosquitoes. This is in agreement with the fact that *Culex* mosquitoes are most likely to lay eggs in stagnant polluted water and their breeding sites are normally near adult feeding areas (Yap et al. 2000). The results of this study demonstrated that the study sites from Kota Bharu (Kelantan), Sitiawan (Perak), Shah Alam (Selangor), and Tuaran (Sabah) were dominated by *Culex* mosquitoes by exhibiting the high BI values. It was suggested that environmental conditions in these study sites favored the infestation by *Culex* mosquitoes. Vector control operations should target these study sites, as high frequency of mosquitoes will increase the risk of disease transmission. In contrast, the study sites from Kuantan (Pahang), Senawang (Negeri Sembilan), Bau (Sarawak), Samarahan 2 (Sarawak), and Ranau (Sabah) exhibited low BI values. In the current study, the number of aquatic predators has not been quantified. Considering that the majority of the sampled habitats in suburban, rural, and remote areas had dragonfly nymphs, water beetles, fish, and tadpoles, one of the possible reasons for the low BI values may be attributed to the presence of these aquatic predators.

The findings of this study also indicate that *Cx. quinquefasciatus* was the most widespread species and well distributed in urban, suburban, rural, and remote areas. It is a cosmopolitan mosquito species distributed in a wide range of larval habitats (Muturi et al. 2007) and is the most common domestic species in urban, suburban, and rural areas, where 53.2–62.7% were reported

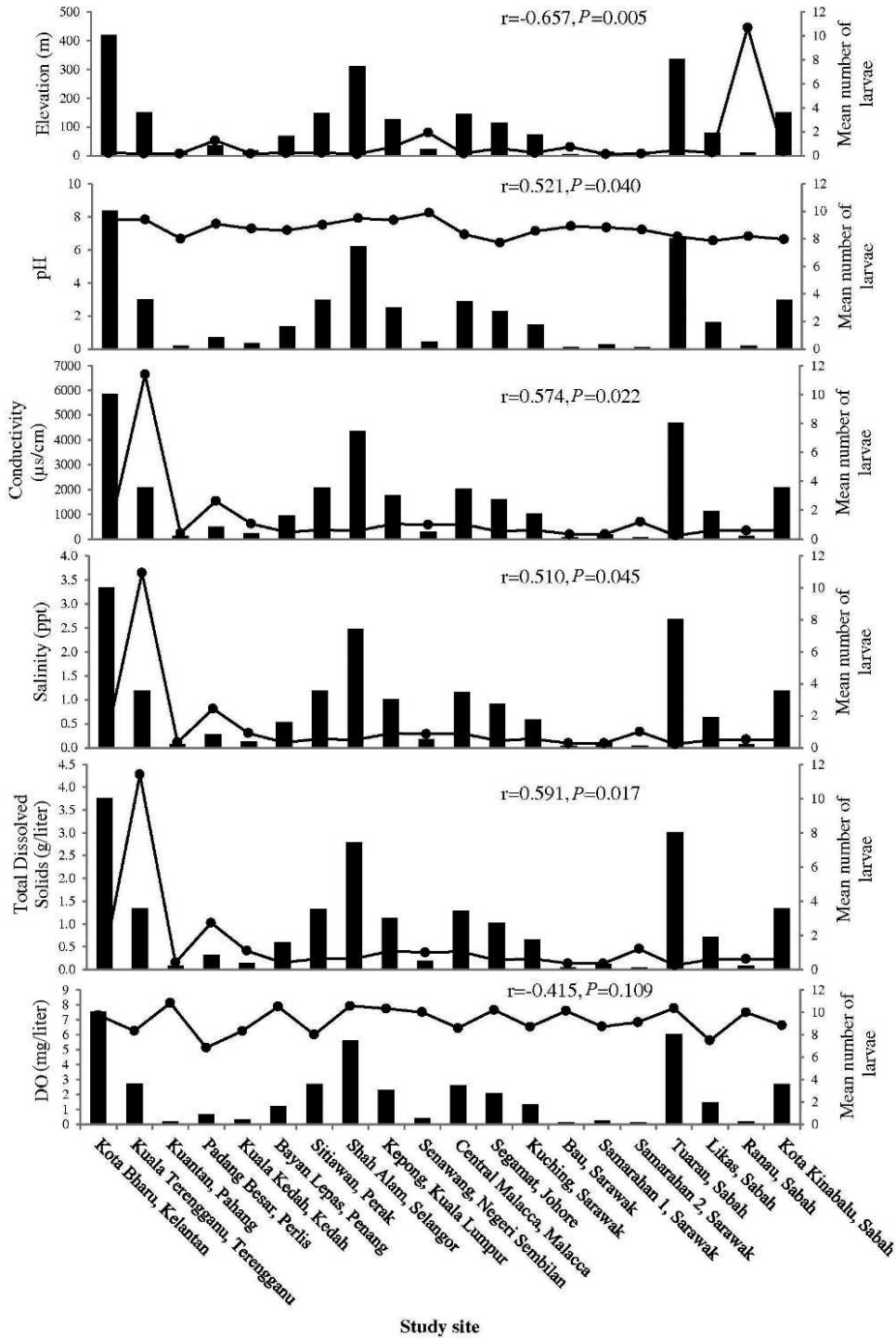

Fig. 3. Correlation between mean number of *Culex* larvae and various habitat characteristics. Bars represent mean number of *Culex* larvae; lines represent habitat characteristics. DO, dissolved oxygen.

to be anthropophilic (Reuben 1992). A wide range of distribution of *Cx. quinquefasciatus* has also been documented in Thailand (Kitvatanachai et al. 2005) and India (Kaliwal et al. 2010). Meanwhile, the distribution of *Cx. vishnui* from the study sites supports the common belief that this species is mainly found in the rural areas. However, *Cx. vishnui* were also found in the

suburban areas of East Malaysia, suggesting that its geographical distribution may contribute to the occurrence of this species in the suburban areas since East Malaysia is mostly surrounded by lowland rainforests and mountain rainforest. *Culex gelidus* mainly occurs in the rural areas, paddy fields, cultivated areas, and pig farms (Tham 2000). The presence of *Cx. gelidus* in the rural areas of Tuaran (Sabah) may be due to intensive pig farming activities in various localities in this area. The pig farms in the Tuaran area not only provide suitable potential breeding sites, but also a blood source for *Cx. gelidus*. This is supported by the observation of Miyagi and Toma (2000) who reported that *Cx. gelidus* preferred to feed on pigs, compared to humans.

Besides the presence of *Culex* mosquitoes, a relatively low number of *Lu. fuscanus*, *An. separatus*, and *Ar. subalbatus* were also detected in stagnant water. It has been reported that *Lu. fuscanus*, *An. separatus*, and *Ar. subalbatus* were commonly found in artificial containers (Chow 1950), swamp areas (Wharton et al. 1963), and tree holes (Lien 1962), respectively. It was suggested that these species were seeking potential breeding sites as a result of environment adaptation.

A significant negative correlation between the mean number of *Culex* larvae and elevation of larval habitat that was noted in the present study corroborated the study of Jacob et al. (2010), where a statistically significant inverse linear relationship between total sampled *Culex* mosquitoes and elevation has been reported. Likewise, De Little et al. (2009) also reported *Aedes* density correlated negatively with elevation.

With regard to water quality assessment, few studies have reported on the relationship between the density of mosquitoes and the physiochemical characteristic of water. Different environmental factors in different locations demonstrated variable results. Minakawa et al. (1999) found that culicine larvae exhibited significant association with pH, and Muturi et al. (2008) reported that *Culex* larvae were positively associated with DO and TDS. In addition, Grillet (2000) reported that the salinity and DO were associated with the spatial distribution of *Anopheles* mosquitoes. Inversely, DO was found to be negatively correlated with the mean number of *Culex* larvae in the current study, which is in agreement with the previous work by Amerasinghe et al. (1995). However, no significant association between the occurrence of mosquito larvae and habitat variables has been documented. It is possible that larval density may be influenced by other habitat characteristics with each contributing some effects or it may be that certain crucial factors have not yet been identified throughout the study sites (Minakawa et al. 1999).

The pH of habitat water ranged from 6.4 to 8.2, revealing that mosquitoes could be found in mildly acidic and alkaline environments. The highest levels of conductivity, TDS, and salinity were recorded from residential areas in Kuala Terengganu (Terengganu), which is surrounded by the sea and periodically receives inflow of seawater, suggesting that salinity tolerance of mosquito larvae occurred in this area as moderate numbers of mosquito larvae, as well as DI and BI values were recorded. However, this finding deserves additional research attention for the investigation of salinity tolerance of mosquito larvae under laboratory and field conditions.

Others habitat characteristics, particularly biotic factors such as the presence of predation, coverage of vegetation, and microorganism identification have not been examined in the current study. It is not clear how other factors affect the female ovipositional behavior. It is possible that these factors may correlate with other habitat characteristics that influence the larval density. However, the current study has identified the potential or actual larval habitats of mosquitoes in residential areas in Malaysia and has demonstrated several correlations between the mosquito density and habitat characteristics. Indeed, a description of their distribution patterns and breeding preferences according to habitat characteristics provide useful baseline data for local authorities in the establishment of action thresholds (e.g., to justify the application of insecticides in accordance with mosquito species and density) and environmental manipulation (e.g., to improve drainage systems) as well as elimination of breeding sources through community participation. A more comprehensive surveillance comprising both biotic and abiotic factors needs to be taken into consideration in the near future to facilitate the management of disease transmission and mosquito control.

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Brief communication (Original)

Co-occurrence of mosquito larvae in stagnant water in residential areas in Malaysia

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Background: The importance of mosquito-borne diseases can be aggravated when there is an occurrence of mixed infestation between the mosquitoes in a habitat. However, there is limited available information on mixed infestation behavior among Malaysian mosquitoes.

Objective: We elucidated the nature of co-occurrence among mosquito species from residential areas in Malaysia.

Methods: Entomological investigation was carried out by using a previously described larval dipping method in 20 residential areas across 11 states and a federal territory (i.e., Kuala Lumpur) in Peninsular Malaysia as well as two states in East Malaysia.

Results: Of 20 study sites, eight study sites exhibited co-occurrence of mosquito larvae, ranging from 1.28% to 50.00%. *Culex quinquefasciatus* was able to breed simultaneously with *Cx. gelidus* (10.00%–50.00%), *Lutzia fuscanus* (2.94%–13.33%), *Cx. vishnui* (5.00%) and *Armigeres subalbatus* (1.28%–3.77%). On the other hand, *Cx. vishnui* was able to breed simultaneously with *Cx. gelidus* (20.00%) and *Lu. fuscanus* (3.33%).

Conclusion: The findings of this study have implications for the development of a better understanding of their mixed infestation behavior and prevention of vector-borne disease transmission from these study sites.

Keywords: *Armigeres*, *Culex*, *Lutzia*, co-occurrence, mixed infestation, Malaysia

To date, 442 species of mosquito representing 20 genera have been recorded in Malaysia [1]. Despite the importance of these mosquitoes in the potential for disease transmission, little is known about their mixed infestation behavior. In recent years, several studies have reported co-occurrence among *Aedes* larvae [2, 3] and co-occurrence between Anopheline and Culicine larvae [4]. However, no report has surfaced thus far pertaining to the mixed infestation behavior among *Culex* sp., *Lutzia* sp. and *Armigeres* sp. in stagnant water in residential areas in Malaysia.

The co-occurrence of more than one species in a habitat implies that they are sharing the same environmental conditions. However, different species of mosquitoes might spread different kinds of mosquito-borne diseases and certain diseases can be transmitted by more than one species of mosquito [1]. The importance of mosquito-borne diseases can be aggravated when there is an occurrence of mixed infestation between the mosquitoes in a habitat. It could be a serious problem in the attempt to assess their roles as vector-borne diseases during the outbreak of disease transmission. Besides, over-reliance of insecticide often causes resistant strains to evolve and different species of mosquitoes might have different rates of resistance development towards various classes of insecticides [5].

The present study focuses on the distribution and the incidence of co-occurrence among mosquito species from the residential areas in Malaysia. The findings of this study have implication for the development of a better understanding of their mixed infestation behavior and prevention of vector-borne disease transmission from these study sites.

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Materials and methods

Entomological investigation was performed using a standardized larval dipping method in 20 residential areas in Malaysia. It has been confirmed that the surveillance period was free from the influence of northeast and northwest monsoon seasons. Mosquito larvae were dipped from stagnant water by using a 330 ml capacity dipper. Standardization of the number of dips in accordance with the surface area of the water body was conducted as follows: number of dips, water surface area (m^2): 1, < 0.25; 2, 0.26–1.0; 3, 1.1–3.0; 4, 3.1–5.0; 5, 5.1–7.0; 6, 7.1–9.0, and so on, as developed by Mendoza et al. [6]. Dips were taken gently with a 2–3 minute pause, to allow the mosquito larvae move freely in the air-water interface. Field-collected larvae were transported to the laboratory and were reared to adulthood for identification. Moribund and dead larvae were subsequently mounted for identification. The mosquito larvae and adults were identified according to taxonomic keys [7, 8].

Results

The percentage of co-occurrence of mosquito larvae obtained from larvae surveillance in Malaysia is demonstrated in **Table 1**. Eight study sites exhibited co-occurrence of mosquito larvae, namely Central Malacca (Malacca), Kota Bharu (Kelantan), Kuching (Sarawak), Ranau (Sabah), Senawang (Negeri Sembilan), Segamat (Johore), Shah Alam (Selangor) and Tuaran (Sabah).

The percentage of co-occurrence according to mosquito species is presented in **Table 2**. *Culex quinquefasciatus* was able to breed simultaneously with *Cx. gelidus* (10.00%–50.00%), *Lu. fuscanus* (2.94%–13.33%), *Cx. vishnui* (5.00%) and *Ar. subalbatus* (1.28%–3.77%). Meanwhile, *Cx. vishnui* was able to breed simultaneously with *Cx. gelidus* (20.00%) and *Lu. fuscanus* (3.33%).

The ratio of mosquito species recorded from co-occurrence dips is presented in **Table 3**. Generally, *Cx. quinquefasciatus* is the dominant species in the majority of dips conducted in Central Malacca (Malacca), Kota Bharu (Kelantan), Segamat (Johore) and Shah Alam (Selangor) by 1.50–10.00-fold. However, *Cx. vishnui* was the dominant species in dips conducted in Tuaran (Sabah) and Kuching (Sarawak) by 1.67–19.00-fold. It is of interest that the drains in Tuaran (Sabah) were inhabited by *Cx. vishnui*, *Cx. gelidus* and *Cx. quinquefasciatus* but the ratio of mixed infestation of these species were low (< 2).

Table 1. Percentage of co-occurrence of mosquito larvae in residential areas in Peninsular and East Malaysia

Study site	*Number of dip conducted	Positive dip		Co-occurrence found in positive dip	
		n	%	n	%
Kota Bharu, Kelantan	121	56	46.28	2	3.57
Kuala Terengganu, Terengganu	197	59	29.95	0	0.00
Kuantan, Pahang	167	19	11.38	0	0.00
Padang Besar, Perlis	123	11	8.94	0	0.00
Kuala Kedah, Kedah	144	17	11.81	0	0.00
Bayan Lepas, Penang	212	10	4.72	0	0.00
Sitiawan, Perak	196	81	41.33	0	0.00
Shah Alam, Selangor	187	34	18.18	1	2.94
Kepong, Kuala Lumpur	162	31	19.14	0	0.00
Senawang, Negeri Sembilan	331	15	4.53	2	13.33
Central Malacca, Malacca	131	53	40.46	2	3.77
Segamat, Johore	267	78	29.21	1	1.28
Kuching, Sarawak	218	30	13.76	1	3.33
Bau, Sarawak	86	5	5.81	0	0.00
Samarahan 1, Sarawak	103	10	9.71	0	0.00
Samarahan 2, Sarawak	113	3	2.65	0	0.00
Tuaran, Sabah	46	20	43.48	7	35.00
Likas, Sabah	162	8	4.94	0	0.00
Ranau, Sabah	53	2	3.77	1	50.00
Kota Kinabalu, Sabah	98	5	5.1	0	0.00
Total	**3117**	**547**	**17.55**	**17**	**3.11**

*Details on larval surveillance have been produced in our previous study [23].

Table 2. Percentage of co-occurrence according to mosquito species

Study site	Positive (n)	Dip (n)										
		CQ n (%)	**CV** n (%)	**CG** n (%)	**AS** n (%)	**LF** n (%)	**AN** n (%)	**CQ+LF** n (%)	**CQ+AS** n (%)	**CQ+CV** n (%)	**CQ+CG** n (%)	**CV+LF** n (%)	**CV+CG** n (%)
Kota Bharu, Kelantan	56	54 (96.43)	0	0	0	0	0	2 (3.57)	0	0	0	0	0
Shah Alam, Selangor	34	33 (97.06)	0	0	0	0	0	1 (2.94)	0	0	0	0	0
Senawang, Negeri Sembilan	15	13 (86.67)	0	0	0	0	0	2 (13.33)	0	0	0	0	0
Central Malacca, Malacca	53	51 (96.23)	0	0	0	0	0	0	2 (3.77)	0	0	0	0
Segamat, Johore	78	77 (98.72)	0	0	0	0	0	0	1 (1.28)	0	0	0	0
Kuching, Sarawak	30	14 (46.67)	15 (50.00)	0	0	0	0	0	0	0	0	1 (3.33)	0
Tuaran, Sabah	20	2 (10.00)	8 (40.00)	3 (15.00)	0	0	0	0	0	1 (5.00)	2 (10.00)	0	4 (20.00)
Ranau, Sabah	2	0	0	0	0	0	1 (50.00)	0	0	0	1 (50.00)	0	0
Total	**288**	**244 (84.71)**	**23 (7.99)**	**3 (1.04)**	**0**	**0**	**1 (0.35)**	**5 (1.74)**	**3 (1.04)**	**1 (0.35)**	**3 (1.04)**	**1 (0.35)**	**4 (1.39)**

CQ = *Cx. quinquefasciatus*, CV = *Cx. vishnui*, CG = *Cx. gelidus*, AS = *Ar. subalbatus*, LF = *Lu. fuscanus*, AN = *An.separatus*

Table 3. Ratio of mosquito species recorded from co-occurrence dips

Study site	CQ : LF	CQ : AS	CQ : CV	CQ : CG	CV : LF	CV : CG
Kota Bharu, Kelantan	3.33 : 1.00	0	0	0	0	0
Shah Alam, Selangor	10.00 : 1.00	0	0	0	0	0
Senawang, Negeri Sembilan	3.25 : 1.00	0	0	0	0	0
Central Malacca, Malacca	0	3.50 : 1.00	0	0	0	0
Segamat, Johore	0	1.50 : 1.00	0	0	0	0
Kuching, Sarawak	0	0	0	0	19.00 : 1.00	0
Tuaran, Sabah	0	0	1.00 : 1.67	1.22 : 1.00	0	1.00 : 1.51
Ranau, Sabah	0	0	0	1.00 : 1.25	0	0

CQ = *Cx. quinquefasciatus*, CV = *Cx. vishnui*, CG = *Cx. gelidus*, AS = *Ar. subalbatus*, LF = *Lu. fuscanus*

Discussion

The co-occurrence of mosquito species regardless of their distribution frequency might be caused by several factors. Interspecific competition between species was the obvious hypothesis tested and has been studied intensively [9-11]. However, several studies have failed to document clear evidence for interspecific competition [12, 13]. It has been suggested that mixed infestation between species might be caused by temporal and spatial variation, rapid and extensive urbanization, difference in fecundity between species, and difference in life-cycle duration between species [12, 14]. It is not surprising to note that *Cx. quinquefasciatus* was able to breed simultaneously with another four species of mosquito in this study as their co-occurrence with another mosquito species have been well-documented around the world. Mixed infestation between *Cx. quinquefasciatus* and *Aedes* mosquitoes has been reported from Malaysia [2] and Brazil [15]. Inversely, co-occurrence of *Cx. quinquefasciatus* with *Cx. nigripalpus* in Florida [16] and *Cx. dolosus affinis* in Brazil [15] has also been elucidated. In Kenya, *Cx. quinquefasciatus* also co-occur with *Anopheles gambiae* [17] and *An. arabiensis* [18]. In the present study, *Cx. vishnui* was found to be able to breed simultaneously with *Cx. quinquefasciatus*, *Cx. gelidus* and *Lu. fuscanus*. It has been reported that *Cx. vishnui* also co-occurs with *Cx. brevipalpis* and *Cx. vishnui* complex in India and Southeast Asia regions, respectively [19-20].

The finding of this study demonstrated that *Ar. subalbatus* only co-occurs with *Cx. quinquefasciatus*. However, previous study has pointed out that *Ar. subalbatus* was also able to breed simultaneously with a large group of mosquitoes (i.e., *Ae. krombeini*, *Ae. albolpictus*, *Cx. uniformis*, *An. elegans*, *Toxorhynchites splendens* and *Tripteroides aranoides*) in Sri Lanka [21].

Co-occurrence of *Lu. fuscanus* with *Cx. quinquefasciatus* and *Cx. vishnui* was recorded in the present study. A previous study found that this species acts as the predator when they co-occurred with *Ae. albopictus*, *An. sinensis*, *Cx. sitiens*, *Cx. quinquefasciatus*, and *Cx. vagans* in China [22]. However, the presence of *Lu. fuscanus*, which occurred in a very low frequency in the present study, did not seemed to be a predator of *Cx. quinquefasciatus* or *Cx. vishnui*.

Conclusion

Although we acknowledge that the present data is insufficient to interpret the mixed population of mosquitoes resulting from factors mentioned in the discussion; nevertheless, the present study has provided the first documented data on the co-occurrence of mosquito larvae among *Culex* sp., *Lutzia* sp. and *Armigeres* sp. in residential areas in Malaysia. The findings of this study indicate that preventive and control measures should be considered proactive when there is an occurrence of mixed infestation between the mosquito species. We certainly do not want to wait till the outbreak of disease transmission that might be spread by different species of mosquitoes. By then, it will be too late to instill remedial action. A more comprehensive study is needed and routine monitoring of vector-borne disease is indispensable in assisting local authorities to improve vector control strategies currently practiced in Malaysia.

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Mitochondrial DNA analyses reveal low genetic diversity in *Culex quinquefasciatus* from residential areas in Malaysia

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Abstract. The present study explored the intraspecific genetic diversity, dispersal patterns and phylogeographic relationships of *Culex quinquefasciatus* Say (Diptera: Culicidae) in Malaysia using reference data available in GenBank in order to reveal this species' phylogenetic relationships. A statistical parsimony network of 70 taxa aligned as 624 characters of the cytochrome c oxidase subunit I (COI) gene and 685 characters of the cytochrome c oxidase subunit II (COII) gene revealed three haplotypes (A1–A3) and four haplotypes (B1–B4), respectively. The concatenated sequences of both COI and COII genes with a total of 1309 characters revealed seven haplotypes (AB1–AB7). Analysis using TCS indicated that haplotype AB1 was the common ancestor and the most widespread haplotype in Malaysia. The genetic distance based on concatenated sequences of both COI and COII genes ranged from 0.00076 to 0.00229. Sequence alignment of *Cx. quinquefasciatus* from Malaysia and other countries revealed four haplotypes (AA1–AA4) by the COI gene and nine haplotypes (BB1–BB9) by the COII gene. Phylogenetic analyses demonstrated that Malaysian *Cx. quinquefasciatus* share the same genetic lineage as East African and Asian *Cx. quinquefasciatus*. This study has inferred the genetic lineages, dispersal patterns and hypothetical ancestral genotypes of *Cx. quinquefasciatus*.

Key words. *Culex quinquefasciatus*, COI, COII, genetic diversity, mitochondrial, Malaysia.

Introduction

Culex is the second largest genus of mosquitoes in Southeast Asia; to date, a total of 94 species of *Culex* have been recorded in Malaysia (Miyagi & Toma, 2000). *Culex quinquefasciatus* (Say), the most abundant species, is a major biting nuisance in Malaysia (Yap *et al*., 2000; Low *et al*., 2012) and a potential vector of bancroftian filariasis in this region (Vythilingam *et al*., 2005).

Although several population studies of *Cx. quinquefasciatus* have been conducted in different localities in Malaysia, using various collection methods, such as light traps (Oli *et al*., 2005), human landing catches (Rohani *et al*., 2008), larval surveillance (Low *et al*., 2012) and container surveillance (Chen *et al*., 2009), none have investigated intraspecific genetic diversity, evolutionary relationships or dispersal patterns in this region.

Globally, the intraspecific genetic diversity of this species has been well characterized in India (Sharma *et al*., 2009, 2010; Mendki *et al*., 2011), Bangladesh (Hasan *et al*., 2009) and South America (Fonseca *et al*., 2000; Morais *et al*., 2012). In 2006, a worldwide genotyping of *Cx. quinquefasciatus*

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covering the continents of Asia, Africa, America, Europe and Australia was documented (Fonseca *et al*., 2006). Important key findings from this study include: (a) isolates of Asian *Cx. quinquefasciatus* (i.e. from India, Indonesia and Japan) and East African *Cx. quinquefasciatus* (i.e. from Kenya) share the same genetic lineage, and (b) there is high genetic diversity in Asian and East African populations of *Cx. quinquefasciatus*. However, this survey tested only one isolate of *Cx. quinquefasciatus* from Southeast Asia, from the archipelago of Indonesia (Fonseca *et al*., 2006), and thus the genetic diversity of Southeast Asian *Cx. quinquefasciatus* may have been underestimated. Therefore, further studies on the genetic diversity of Southeast Asian isolates, including those of Malaysia, are required.

As far as the application of genetic markers is concerned, mitochondrial DNA is the most widely used marker for the study of molecular ecology in animal taxa (Simon *et al*., 1994; Norris, 2002; Pramual *et al*., 2005). The assessment of genetic variation within a species has indicated that animal mitochondrial DNA is an ideal molecular marker as a result of its uniparental inheritance, lack of recombination and higher rate of mutation (Lowe *et al*., 2004). The literature suggests that, among the mitochondrial DNA markers, both the COI and COII genes have been reliable genetic markers in studies of mosquito population genetics (Mukabayire *et al*., 1999; Walton *et al*., 2000; Chen *et al*., 2004). With regard to phylogeographical studies of *Cx. quinquefasciatus*, investigations of the mitochondrial COI and ND4 genes have been carried out in South America (Morais *et al*., 2012). In addition, COII and 16S ribosomal RNA (16S rRNA) genes have been characterized in Bangladesh and India. Previous studies revealed a lack of population genetic structure of *Cx. quinquefasciatus* in South America and Bangladesh. This has been inferred from the sequences of COI (four haplotypes), COII (two haplotypes) and ND4 (one haplotype) (Hasan *et al*., 2009; Morais *et al*., 2012). Inversely, the 16S rRNA gene demonstrated that Indian *Cx. quinquefasciatus* was genetically diverse (Sharma *et al*., 2010).

Given the variability and resolution of mitochondrial markers, a comparative assessment of the genetic structure of Malaysian *Cx. quinquefasciatus* was attempted in the present study by using COI, COII, 16S rRNA and NADH dehydrogenase subunit 5 (ND5) genes. Preliminary data showed that both COI and COII genes were more variable and demonstrated higher resolution than the 16S rRNA and ND5 genes (Low *et al*., 2012, unpublished data). The present study aimed to examine intraspecific genetic diversity, dispersal patterns and phylogeographic relationships based on COI and COII sequences of *Cx. quinquefasciatus* from 11 states and a federal territory (i.e. Kuala Lumpur) in peninsular Malaysia, and two states in East Malaysia, which is separated from the peninsula by the South China Sea. In conjunction with the COI and COII sequences from Malaysian isolates, published sequences available in GenBank were used to reveal the phylogenetic relationships in populations from different countries. A better understanding of the genetic lineages of this species could be utilized in the implementation of strategic measures in vector control programmes in Malaysia. This is the first study to examine the population genetic structure of *Cx. quinquefasciatus* sampled from residential areas in Malaysia.

Materials and methods

Mosquito specimens

As dengue is the main vector-borne disease in Malaysia, specific vector control activities mainly target *Aedes* (*Stegomyia*) species (Diptera: Culicidae) rather than *Culex* species. Therefore, study sites were selected according to vector control programme activities in areas in which frequent reports of dengue cases resulted in fogging. It was speculated that mosquito control programmes would also exert selective pressure on *Cx. quinquefasciatus* in these study sites.

Mosquito specimens were collected using the dipping method from 14 selected residential areas across all states in Malaysia (Table 1). Field-collected larvae and pupae were reared to adults for identification. Adult mosquitoes were identified using illustrated keys (Rattanarithikul *et al*., 2005). Adult mosquitoes were then frozen and stored at $-80\,^{\circ}$C prior to DNA extraction. In the present study, a total of 70 adult females of *Cx. quinquefasciatus*, comprising five individuals from each of the 14 study sites, were randomly selected from a previous nationwide collection (of 6441 individuals) (Low *et al*., 2012). This selection procedure was adopted to avoid the sampling bias that may arise if samples are collected from a single source (Latch & Rhodes, 2006).

DNA extraction

Prior to DNA extraction, abdomens were dissected from the mosquito samples to avoid contamination. DNA was extracted from each specimen using the i-genomic CTB DNA Extraction Mini Kit™ (iNtRON Biotechnology, Inc., Seongnam, South Korea). All isolation steps were performed according to the manufacturer's instructions.

Polymerase chain reaction

A subset of 20 representative individual samples was screened for genetic variation targeting the COI, COII, 16S rRNA and ND5 genes. Preliminary data revealed that there was no site variation in 16S rRNA and ND5 sequences, but that both COI and COII genes were more variable and demonstrated higher resolution. Therefore, COI and COII sequences were used as mitochondrial markers in the present study.

The amplification of extracted genomic DNA was conducted using mitochondrial primers of COI from Kumar *et al*. (2007) (forward primer, 5′-3′; reverse primer, 5′-3′) and COII from Ndo *et al*. (2010) (forward primer, 5′-GGA TTT GGA AAT TGA TTA GTT CCT T-3′; reverse primer, 5′-AAA AAT TTT AAT TCC AGT TGG AAC AGC-3′). The amplification of COI and COII regions was performed in a final volume of 50 μL containing 5 μL 10× buffer, 2.5 mM of each dNTP, 10 pmol of each forward and reverse primer, 1.5 U

Table 1. Geographical description of study sites in Malaysia.

Malaysia	Region	State	District	Study site	Landscape
Peninsular	East Coast	Kelantan	Kota Bharu	Taman Guru	Suburban
		Terengganu	Kuala Terengganu	Kampung Simpang Empat	Rural
		Pahang	Kuantan	Taman Chenderawasih	Suburban
	Northern	Perlis	Padang Besar	Taman Singgahsana	Rural
		Kedah	Kuala Kedah	Taman Selat	Suburban
		Penang	Bayan Lepas	Taman Bayan Baru	Urban
		Perak	Sitiawan	Taman Bunga Ros	Suburban
	Central	Selangor	Shah Alam	Section 17	Urban
		Kuala Lumpur	Kepong	Kepong Baru	Urban
	Southern	Negeri Sembilan	Senawang	Taman Marida	Suburban
		Malacca	Central Malacca	Kampung Pengkalan Rama Pantai	Rural
		Johore	Segamat	Segamat Baru	Suburban
East Malaysia	West	Sarawak	Kuching	RPR Batu Kawa	Suburban
	East	Sabah	Kota Kinabalu	Taman Kepayan	Suburban

Taq polymerase (iNtRON Biotechnology, Inc.) and 25–50 ng genomic mosquito DNA. Polymerase chain reaction (PCR) was carried out using the MyCycler™ Thermal Cycler (580BR 7200; Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.). The PCR conditions of COI included an initial denaturation of 95 °C for 5 min, followed by five cycles of 94 °C for 40 s (denaturation), 45 °C for 1 min (annealing) and 72 °C for 1 min (extension), and 35 cycles of 94 °C for 40 s (denaturation), 51 °C for 1 min (annealing) and 72 °C for 1 min (extension), and a final extension at 72 °C for 10 min. For COII, PCR conditions included an initial denaturation of 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing) and 72 °C for 45 s (extension), and a final extension at 72 °C for 10 min.

DNA purification

The amplified fragments were electrophoresed on 2% agarose gel pre-stained with SYBR Safe™ (Invitrogen Corp., Carlsbad, CA, U.S.A.). The PCR products were purified with MEGAquick-spin™ PCR & Agarose Gel DNA Extraction System (iNtRON Biotechnology, Inc.). Purified PCR products were sent to a commercial company for DNA sequencing. Samples were sequenced using BigDyeH Terminator 3.1 Sequencing Kit™ and analysed using an ABI PRISM 377 Genetic Analyser™ (Applied Biosystems, Inc., Foster City, CA, U.S.A.).

DNA sequences alignment

Data on the nucleotide sequences of the COI and COII genes of Malaysian *Cx. quinquefasciatus* were deposited in GenBank under the accession numbers JQ716469–JQ716608. Sequencing data were analysed and edited using ChromasPro 1.5® (Technelysium Pty Ltd, Brisbane, Qld, Australia) and BioEdit 7.0.9.0.® (Hall, 1999). The partial COI and COII sequences were preliminarily aligned using ClustalX® (Thompson *et al*., 1997) and subsequently aligned manually.

Haplotype network reconstruction

Malaysian Cx. quinquefasciatus *haplotype.* The genetic diversity or haplotype networks of *Cx. quinquefasciatus* were analysed using TCS 1.13® (Clement *et al*., 2000) to calculate the minimum number of mutational steps by which the sequences could be joined with > 95% confidence. The aligned COI and COII sequences consisted of 624 bp and 685 bp, respectively. The multiple sequences of both COI and COII were concatenated and yielded a total length of 1309 bp.

Comparison of Malaysian Cx. quinquefasciatus *with other* Cx. quinquefasciatus *from GenBank).* The genetic diversity or haplotype networks of *Cx. quinquefasciatus* were analysed using TCS 1.13® (Clement *et al*., 2000) to calculate the minimum number of mutational steps by which the sequences could be joined with > 95% confidence. Some sequences of *Cx. quinquefasciatus* were trimmed in length in order to ensure equal lengths of alignment for the purposes of comparison; the final lengths of the aligned COI and COII sequences used for analysis were 434 bp and 661 bp, respectively. The COI and COII sequences deposited in GenBank that did not correspond in length or region to the sequences of Malaysian *Cx. quinquefasciatus* generated in this study were discarded.

Genetic divergence

Uncorrected (*p*) pairwise genetic distances were estimated using PAUP* Version 4.0b10® (Swofford, 2002) to assess the levels of variation in the concatenated sequences of both COI and COII genes among the representative samples.

Phylogenetic analyses

Similar sets of COI and COII sequences of *Cx. quinquefasciatus* used in haplotype analysis were aligned with other sequences of *Culex* taxa obtained from

GenBank and subjected to maximum likelihood (ML), maximum parsimony (MP), Bayesian inference (BI) and neighbour-joining (NJ) analyses.

Maximum likelihood analysis was performed using Treefinder® Version October 2008 (Jobb *et al*., 2004). Bayesian inference analysis was performed using MrBayes 3.1.2® (Huelsenbeck & Ronquist, 2001). The best fit nucleotide substitution model was determined using KAKUSAN® Version 3 (Tanabe, 2007), which also generates input files for ML and BI. Best fit models were evaluated using the corrected Akaike information criterion (AIC) (Akaike, 1973; Shono, 2000) for ML and the Bayesian information criterion (BIC), with significance determined by chi-squared analysis. The best selected model for COI was a general time-reversible (GTR) model of DNA evolution with a gamma-shaped parameter (G), whereas the best selected model for COII was a J1 model with a gamma-shaped parameter (G). Maximum likelihood analysis was performed with 1000 bootstrap replicates. Two parallel runs were performed in MrBayes analysis using four chains of Markov chain Monte Carlo (MCMC). Four million MCMC generations were run, with convergence diagnostics calculated every 1000th generation to monitor the stabilization of log likelihood scores. Trees in each chain were sampled every 100th generation. Likelihood scores were stabilized at 650 000 generations for COI and 550 000 generations for COII. A 50% majority rule consensus tree was generated from the sampled trees after the first 20% had been discarded. Maximum parsimony and NJ analyses were performed using PAUP* 4.0b10® (Swofford, 2002). The MP tree was constructed using the heuristic search option, 100 random sequences additions, tree bisection reconnection (TBR) branch swapping, and unordered and unweighted characters. Bootstrap percentage (BP) was computed with 1000 replications. Neighbour-joining bootstrap values were estimated using 1000 replicates with Kimura's two-parameter model of substitution (K2P distance) evolution model. *Aedes albopictus* (*Stegomyia albopicta*) (HQ398901) and *Ae. albopictus* (HQ398974) were used as outgroups for the construction of phylogenetic trees of COI and COII, respectively.

Results

Haplotype network reconstruction

Malaysian Cx. quinquefasciatus *haplotype.* Based on morphological features, all adult females were unambiguously identified and no aberrant characters were found. The partial regions of COI (positions 263–886) and COII (positions 1–685) were successfully sequenced from 70 individual samples. A statistical parsimony network of 70 taxa aligned as 624 characters of the COI gene and 685 characters of the COII gene revealed three haplotypes (A1–A3) and four haplotypes (B1–B4), respectively (Table 2).

For concatenated sequences, a total of 1309 characters of both COI and COII genes revealed seven haplotypes (AB1–AB7) (Fig. 1). Results indicated that haplotype AB1 was the common ancestor and the most widespread haplotype based on its prevalence in Malaysia. Two haplotypes (AB4 and AB6) were discovered in Kuantan (Pahang) with the absence of the common ancestor (AB1). There was a substitution of guanine to adenine at positions 95, 527, 731 and 770 for haplotype AB6 from a majority of the localities, AB7 from Kota Kinabalu (Sabah), AB2 from Bayan Lepas (Penang) and AB4 from Kuantan (Pahang). Haplotype AB3 from Senawang (Negeri Sembilan) consisted of two base changes at which a guanine was substituted by adenine at positions 95 and 731. A substitution of adenine for guanine at position 1033 was observed in haplotype AB5 from Shah Alam (Selangor) and Kepong (Kuala Lumpur).

Comparison of Malaysian Cx. quinquefasciatus *with other* Cx. quinquefasciatus *in GenBank.* For comparison purposes, COI and COII sequences of *Cx. quinquefasciatus* from other countries were obtained from GenBank (Table 3). Four haplotypes (AA1–AA4) were revealed when COI sequences of *Cx. quinquefasciatus* from Uganda, India, Iran and Thailand were compared with those of Malaysian *Cx. quinquefasciatus* (Fig. 2A). There was a substitution of guanine to adenine at position 95 in haplotype AA2. Haplotype AA3 from Iran showed two base changes at which a guanine was substituted by adenine at positions 8 and 95. Haplotype AA4 from Thailand showed three base changes at which an adenine was substituted by guanine at positions 257 and 386, and thymine was substituted for cytosine at position 425.

Moreover, nine haplotypes (BB1–BB9) were revealed when COII sequences of *Cx. quinquefasciatus* from Bangladesh, China, Taiwan and Thailand were compared with those of Malaysian *Cx. quinquefasciatus* (Fig. 2B). In Chinese sources, there was a substitution of guanine to thymine at position 22 and a substitution of thymine to cytosine at position 404, as revealed in haplotype BB2. A substitution of adenine to guanine at position 384 was revealed in haplotype BB3 from Shah Alam (Selangor) and Kepong (Kuala Lumpur). In haplotype BB4, a guanine was substituted by adenine at position 82. The haplotype BB5 from China revealed three mutation changes: a guanine to adenine at position 385; a thymine to guanine at position 629, and an insertion of adenine at position 638. Haplotype BB6 from China also revealed two mutation changes: a guanine to adenine at position 121, and an insertion of adenine at position 638. Substitutions of guanine to adenine at position 121, cytosine to adenine at position 531 and adenine to guanine at position 582 were detected in haplotype BB7 from Kuantan (Pahang), haplotype BB8 from Thailand and haplotype BB9 from Bangladesh, respectively. Both COI and COII inferred that haplotypes AA1 and BB1 were the common ancestors and the most widespread haplotypes in populations in a majority of the countries investigated (Fig. 2A, B).

Genetic divergence

The uncorrected '*p*' distances between different haplotype of *Cx. quinquefasciatus* based on concatenated sequences of both COI and COII genes are summarized in Table 4.

Table 2. Haplotype of *Culex quinquefasciatus* by COI and COII genes.

Study site	*n*	ID no.	COI haplotype	GenBank accession no.	COII haplotype	GenBank accession no.
Kota Bharu	5	ISBUM 001	A1	JQ716488	B1	JQ716577
(Kelantan)		ISBUM 002	A1	JQ716487	B1	JQ716572
		ISBUM 003	A1	JQ716492	B1	JQ716581
		ISBUM 004	A1	JQ716507	B1	JQ716605
		ISBUM 005	A2	JQ716526	B1	JQ716559
Kuala Terengganu	5	ISBUM 006	A2	JQ716489	B1	JQ716546
(Terengganu)		ISBUM 007	A2	JQ716535	B1	JQ716574
		ISBUM 008	A1	JQ716518	B1	JQ716586
		ISBUM 009	A2	JQ716510	B1	JQ716594
		ISBUM 010	A2	JQ716506	B1	JQ716596
Kuantan	5	ISBUM 011	A2	JQ716481	B1	JQ716545
(Pahang)		ISBUM 012	A1	JQ716476	B3	JQ716548
		ISBUM 013	A2	JQ716485	B1	JQ716551
		ISBUM 014	A2	JQ716504	B1	JQ716582
		ISBUM 015	A1	JQ716529	B3	JQ716585
Padang Besar	5	ISBUM 016	A1	JQ716534	B1	JQ716604
(Perlis)		ISBUM 017	A1	JQ716469	B1	JQ716601
		ISBUM 018	A1	JQ716499	B1	JQ716561
		ISBUM 019	A1	JQ716512	B1	JQ716584
		ISBUM 020	A1	JQ716517	B1	JQ716566
Kuala Kedah	5	ISBUM 021	A1	JQ716536	B1	JQ716580
(Kedah)		ISBUM 022	A1	JQ716537	B1	JQ716541
		ISBUM 023	A1	JQ716483	B1	JQ716575
		ISBUM 024	A1	JQ716524	B1	JQ716602
		ISBUM 025	A1	JQ716532	B1	JQ716593
Bayan Lepas	5	ISBUM 026	A1	JQ716478	B1	JQ716600
(Penang)		ISBUM 027	A1	JQ716486	B4	JQ716564
		ISBUM 028	A1	JQ716484	B1	JQ716557
		ISBUM 029	A1	JQ716474	B1	JQ716608
		ISBUM 030	A1	JQ716508	B1	JQ716543
Sitiawan	5	ISBUM 031	A2	JQ716502	B1	JQ716549
(Perak)		ISBUM 032	A1	JQ716516	B1	JQ716542
		ISBUM 033	A1	JQ716528	B1	JQ716544
		ISBUM 034	A2	JQ716533	B1	JQ716571
		ISBUM 035	A2	JQ716527	B1	JQ716583
Shah Alam	5	ISBUM 036	A1	JQ716511	B1	JQ716589
(Selangor)		ISBUM 037	A2	JQ716503	B1	JQ716599
		ISBUM 038	A1	JQ716475	B2	JQ716590
		ISBUM 039	A2	JQ716505	B1	JQ716588
		ISBUM 040	A1	JQ716472	B1	JQ716554
Kepong	5	ISBUM 041	A1	JQ716509	B2	JQ716547
(Kuala Lumpur)		ISBUM 042	A2	JQ716500	B1	JQ716607
		ISBUM 043	A1	JQ716530	B1	JQ716560
		ISBUM 044	A1	JQ716479	B1	JQ716565
		ISBUM 045	A1	JQ716477	B1	JQ716573
Senawang	5	ISBUM 046	A2	JQ716501	B4	JQ716555
(Negeri Sembilan)		ISBUM 047	A2	JQ716490	B1	JQ716579
		ISBUM 048	A1	JQ716482	B1	JQ716578
		ISBUM 049	A2	JQ716514	B1	JQ716603
		ISBUM 050	A2	JQ716515	B1	JQ716567
Central Malacca	5	ISBUM 051	A1	JQ716497	B1	JQ716558
(Malacca)		ISBUM 052	A1	JQ716519	B1	JQ716553
		ISBUM 053	A1	JQ716493	B1	JQ716563
		ISBUM 054	A1	JQ716523	B1	JQ716569
		ISBUM 055	A2	JQ716522	B1	JQ716587
Segamat	5	ISBUM 056	A1	JQ716513	B1	JQ716552
(Johore)		ISBUM 057	A1	JQ716471	B1	JQ716540
		ISBUM 058	A2	JQ716480	B1	JQ716550
		ISBUM 059	A1	JQ716470	B1	JQ716570

Table 2. Continued.

Study site	*n*	ID no.	COI haplotype	GenBank accession no.	COII haplotype	GenBank accession no.
		ISBUM 060	A1	JQ716473	B1	JQ716568
Kuching	5	ISBUM 061	A1	JQ716496	B1	JQ716591
(Sarawak)		ISBUM 062	A1	JQ716494	B1	JQ716576
		ISBUM 063	A1	JQ716498	B1	JQ716562
		ISBUM 064	A1	JQ716531	B1	JQ716597
		ISBUM 065	A1	JQ716520	B1	JQ716606
Kota Kinabalu	5	ISBUM 066	A1	JQ716495	B1	JQ716539
(Sabah)		ISBUM 067	A1	JQ716538	B1	JQ716592
		ISBUM 068	A1	JQ716491	B1	JQ716556
		ISBUM 069	A3	JQ716525	B1	JQ716595
		ISBUM 070	A1	JQ716521	B1	JQ716598

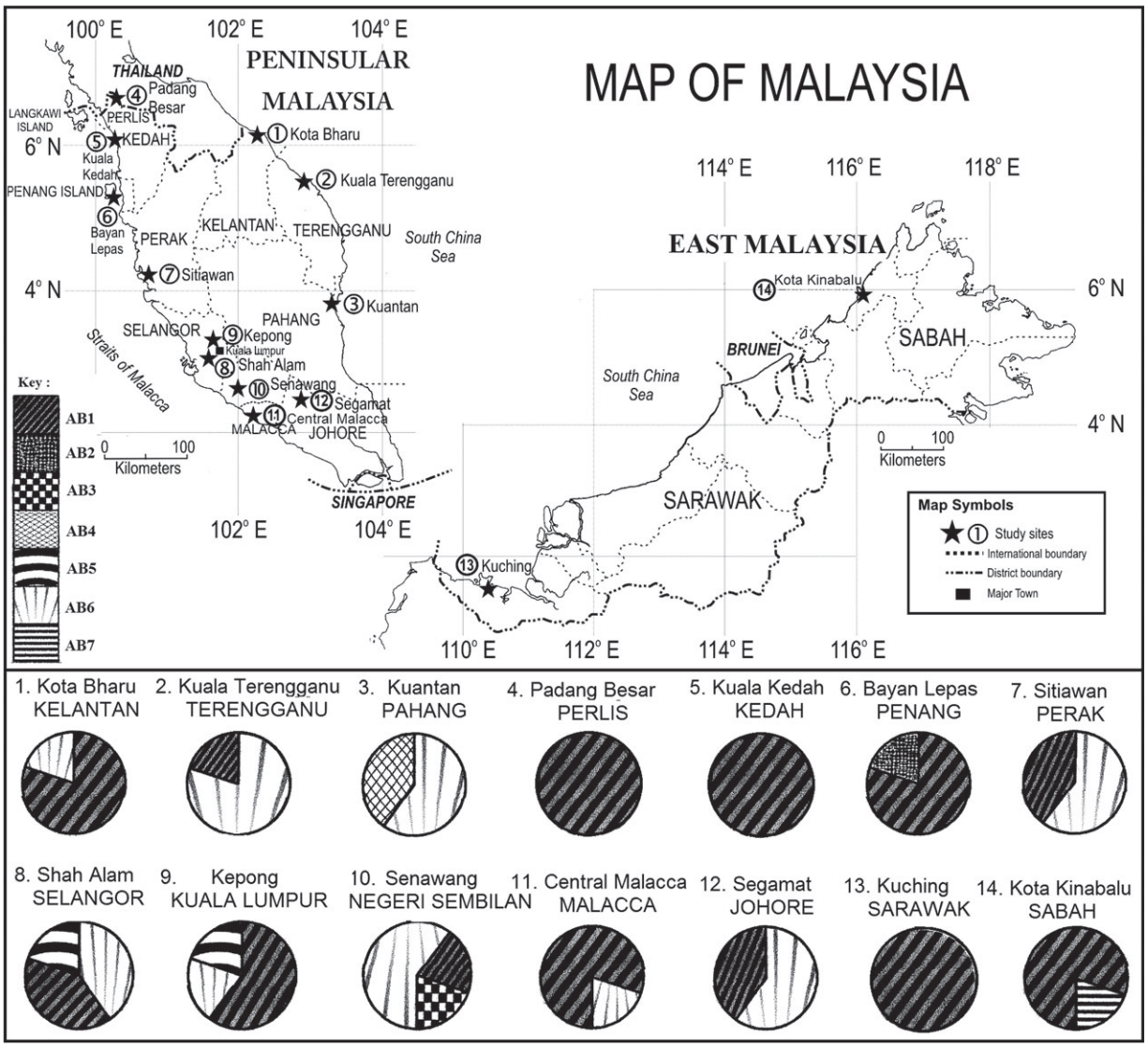

Fig. 1. Haplotype distribution (AB1–AB7) of concatenated sequences of COI and COII for *Culex quinquefasciatus* in Malayasia.

Table 3. Haplotype of *Culex quinquefasciatus* from other countries by COI and COII genes.

Country	*n*	GenBank accession no.	Haplotype
COI gene			
Uganda	9	GQ165791	AA1
		GQ165792	AA1
		GQ165793	AA1
		GQ165794	AA1
		GQ165795	AA1
		GQ165796	AA1
		GQ165797	AA1
		GQ165798	AA1
		GQ165807	AA1
India	5	FN395201	AA1
		FN395202	AA1
		FN395204	AA1
		FN395205	AA1
		AY729977	AA1
Iran	3	FJ210901	AA3
		FJ210909	AA1
		FJ210910	AA1
Thailand	1	HQ398883	AA4
COII gene			
Bangladesh	2	EU014281	BB1
		EU014282	BB9
China	3	AY949854	BB5
		AY949855	BB6
		AF325716	BB2
Taiwan	1	L34351	BB1
Thailand	1	HQ398945	BB8

Phylogenetic analyses

The aligned partial sequences of COI consisted of 434 sites, of which 315 characters were constant, 84 characters were parsimony informative and 35 characters were parsimony uninformative. Maximum parsimony analysis demonstrated a consistency index of 0.5618 and retention index of 0.5753. The aligned partial sequences of COII consisted of 662 sites, of which 514 characters were constant, 67 characters were parsimony informative and 81 were parsimony uninformative. Maximum parsimony analysis demonstrated a consistency index of 0.7149 and retention index of 0.7186.

Four phylogenetic analyses produced phylogenetic trees with the same topology but with different bootstrap support values (Figs 3 and 4). Only ML trees were presented for the sequences of COI and COII.

Cytochrome c oxidase subunit I (COI)

The COI ML tree comprised two main groups (Fig. 3). The first group consisted of *Culex nigropunctatus* with no bootstrap support value. The second group, with no bootstrap to low bootstrap support values (MP = 58%, BI = 51%), was further divided into two main subgroups. The first subgroup consisted of *Culex tritaeniorhynchus*, which is the basal group and was supported with no bootstrap to high bootstrap support values (ML = 54%, BI = 92%, NJ = 50%). The second subgroup consisted of *Cx. quinquefasciatus*, *Culex fuscocephala*, *Culex bitaeniorhynchus*, *Culex gelidus* and *Culex rubithoracis* with no bootstrap support. The second subgroup was further divided into two main clades: clade 1 and clade 2. Clade 1 consisted of *Cx. gelidus* and *Cx. rubithoracis* with no bootstrap support. Isolates of Indian and Thai *Cx. gelidus* were grouped in a monophyletic clade and supported with high to full bootstrap values (ML = 99%, MP = 100%, BI = 100%, NJ = 100%). Clade 2 was further divided into two subclades. Subclade 1 consisted of *Cx. fuscocephala* and *Cx. bitaeniorhynchus*. Isolates of Indian and Thai *Cx. fuscocephala* were grouped in a monophyletic clade and supported with full bootstrap values (ML = 100%, MP = 100%, BI = 100%, NJ = 100%). Subclade 2 consisted of two main groups: (a) *Cx. quinquefasciatus* from Uganda, India, Iran, Thailand and Malaysia (haplotypes AA1 and AA4), supported by low bootstrap values (ML = 64%, MP = 63%, BI = 68%, NJ = 65%), and (b) *Cx. quinquefasciatus* from Malaysia and Iran (haplotypes AA2 and AA3), with no bootstrap support values.

Cytochrome c oxidase subunit II (COII)

The COII ML tree comprised two main groups (Fig. 4). The first group with no bootstrap support consisted of *Cx. fuscocephala*, *Cx. gelidus*, *Cx. tritaeniorhynchus* and *Cx. bitaeniorhynchus*. *Culex fuscocephala* and *Cx. gelidus* were the basal species and showed a sister relationship to *Cx. tritaeniorhynchus* and *Cx. bitaeniorhynchus*, with moderate to high bootstrap support (ML = 86%, MP = 79%, BI = 97%, NJ = 73%). Isolates of *Cx. bitaeniorhynchus* from Thailand and Vietnam were grouped in a monophyletic clade supported with high to full bootstrap values (ML = 90%, MP = 98%, BI = 100%, NJ = 100%). Another main group was divided into two subgroups. Subgroup 1 comprised *Cx. nigropunctatus* and *Cx. rubithoracis* with moderate to high bootstrap support values (ML = 72%, MP = 70%, BI = 92%, NJ = 72%). Subgroup 2 consisted of all *Cx. quinquefasciatus* from various localities, with full bootstrap support values (ML = 100%, MP = 100%, BI = 100%, NJ = 100%). *Culex quinquefasciatus* from Thailand, haplotype BB8 was the most basal member and showed a sister relationship to the other *Cx. quinquefasciatus*, which was supported with low to no bootstrap support (ML = 53%). The isolates (AY949854 and ISBUM012) from China and Malaysia (haplotypes BB5 and BB7) differed from other haplotypes with low to high bootstrap support values (ML = 64%, MP = 63%, BI = 96%, NJ = 62%).

Discussion

Analysis by TCS revealed that haplotype AB1 was the most widespread haplotype of *Cx. quinquefasciatus* as a result of its dispersion in Malaysia (Fig. 1). *Culex quinquefasciatus* from Shah Alam (Selangor), Kepong (Kuala Lumpur) and Senawang (Negeri Sembilan) demonstrated higher divergence with the identification of three different haplotypes. Inversely, the least

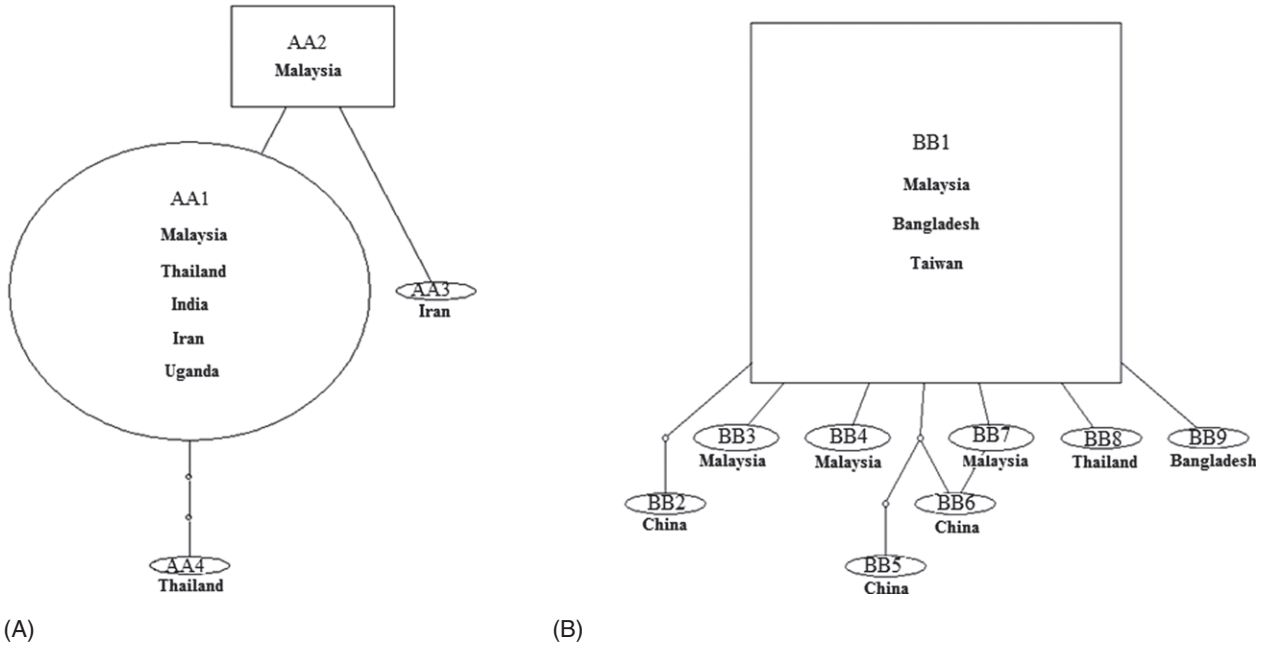

Fig. 2. Statistical parsimony networks for (A) COI and (B) COII haplotypes of *Culex quinquefasciatus* in Malaysia and other countries. Lines represent parsimonious connections between haplotypes with probabilities of $> 95\%$, with each representing one mutational step. Small circles indicate missing haplotypes. Relative sizes of squares and ovals indicate haplotype frequency. Haplotypes AA1 and BB1 were inferred as the hypothetical ancestral haplotypes, respectively.

genetic diversity was detected in *Cx. quinquefasciatus* from Padang Besar (Perlis), Kuala Kedah (Kedah) and Kuching (Sarawak), in which only one haplotype (AB1) was recorded. The present authors propose that haplotype AB1 was the common ancestor of *Cx. quinquefasciatus* and evolved over time into the various haplotypes, namely, AB2, AB3, AB4, AB5, AB6 and AB7, in order to adapt to environmental changes and consequently became distributed across all states in Malaysia. Furthermore, the genetic diversity of Malaysian *Cx. quinquefasciatus* was considered to be extremely low as only three haplotypes were revealed by COI and four haplotypes were revealed by COII. Likewise, a lack of population genetic structure in this species has been observed in South America (Morais *et al*., 2012) and Bangladesh (Hasan *et al*., 2009), where only four and two haplotypes were revealed by COI and COII, respectively. Nonetheless, the previous study conducted by Fonseca *et al*. (2006) revealed high genetic variability in Asian *Cx. quinquefasciatus* (i.e. in India, Indonesia and Japan), which contrasts with the levels of variability in Malaysian isolates.

The genetic distance based on concatenated sequences of both COI and COII genes ranged from 0.00076 to 0.00229. A relatively low genetic distance between Malaysian *Cx. quinquefasciatus* from various localities contrasted sharply with that in Indian *Cx. quinquefasciatus*, in which the highest genetic distance of 0.50117 based on 16 rRNA sequences was recorded (Sharma *et al*., 2010). The low genetic distance between the haplotypes indicated that the genetic diversity of Malaysian *Cx. quinquefasciatus* was low compared with that of Indian *Cx. quinquefasciatus*. Although the markers used by the present authors differed from those used by Sharma *et al*. (2010), preliminary screening using the same 16S rRNA on 20 samples showed no genetic distance among Malaysian isolates. This is likely to be attributable to the field sampling

Table 4. Uncorrected '*p*' distance matrix between Malaysian *Culex quinquefasciatus* based on concatenated sequences of both COI and COII genes.

Haplotype	1	2	3	4	5	6
1 AB1						
2 AB2	0.00076					
3 AB3	0.00153	0.00076				
4 AB4	0.00076	0.00153	0.00229			
5 AB5	0.00076	0.00153	0.00229	0.00153		
6 AB6	0.00076	0.00153	0.00076	0.00153	0.00153	
7 AB7	0.00076	0.00153	0.00229	0.00153	0.00153	0.00153

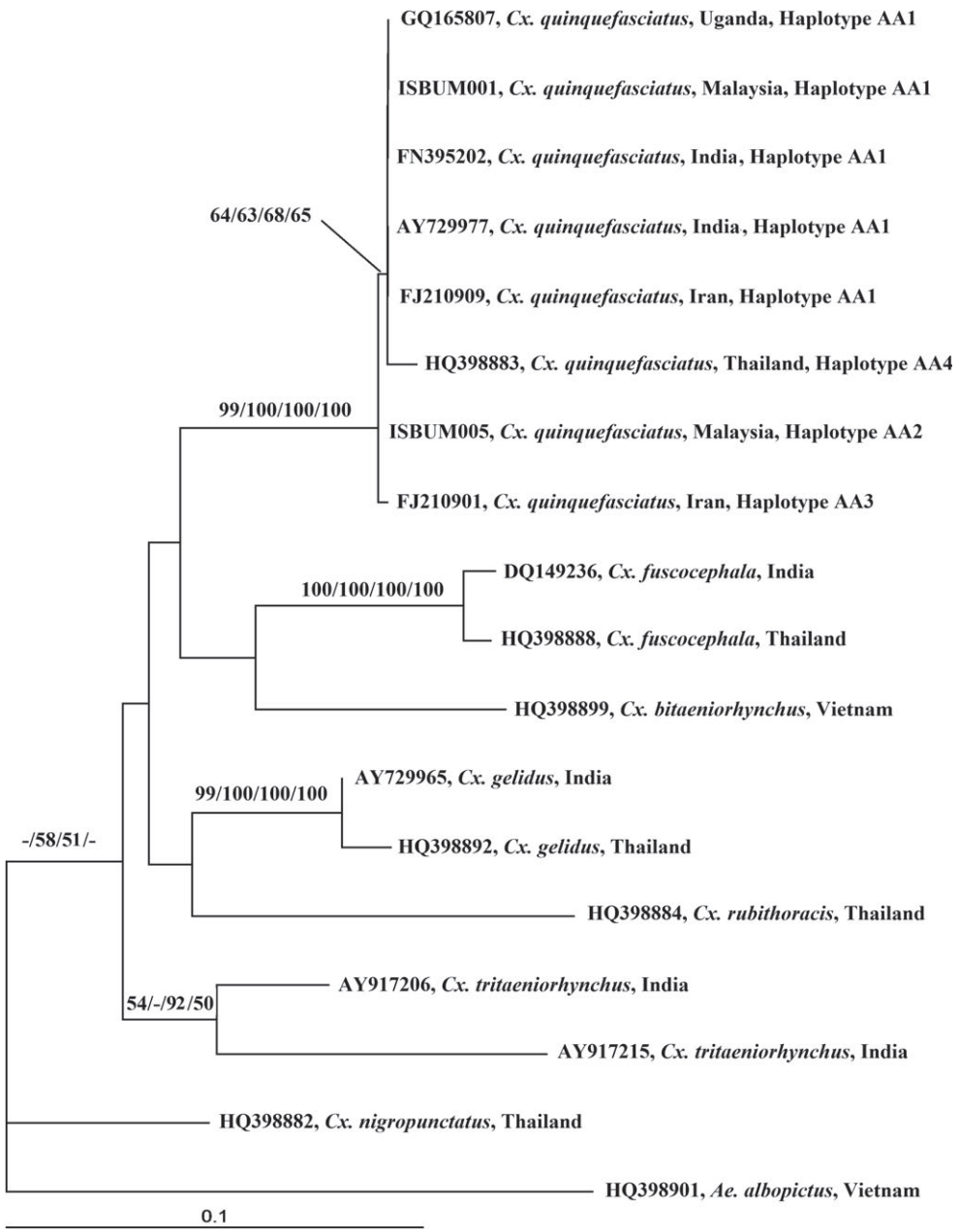

Fig. 3. Maximum likelihood phylogeny tree of *Culex* taxa based on COI sequences. Bootstrap [maximum likelihood (ML)/maximum parsimony (MP)/Bayesian inference (BI)/neighbour-joining (NJ)] values are shown at the branches. Bar indicates substitutions per site.

protocol used in the present study, in which all collections were obtained within 1 year at a single time-point in each location across the country. The genetic diversity might have been higher if sampling had been conducted over a longer period of time, but not as inexplicably high as that observed by Sharma *et al*. (2010). However, this speculation can only be verified by conducting additional sampling efforts over a longer period in Malaysia.

With respect to phylogenetic analyses, both COI and COII sequences of *Cx*. *quinquefasciatus* revealed that there were no distinct genetic lineages between populations in Malaysia and those in a neighbouring country (i.e. Thailand) or between isolates from other Asian countries (i.e. India, Iran, China, Bangladesh and Taiwan) and East Africa (i.e. Uganda). It is possible that a phylogenetic relationship could not be demonstrated because the COI and COII sequences deposited in GenBank were limited and some of the sequences were discarded in favour of sequences that corresponded in length or region to the sequences of Malaysian *Cx*. *quinquefasciatus* generated in this study. However, this study has demonstrated that there is a lack of phylogeographic relationship between the haplotype and country of origin. Likewise, a previous study

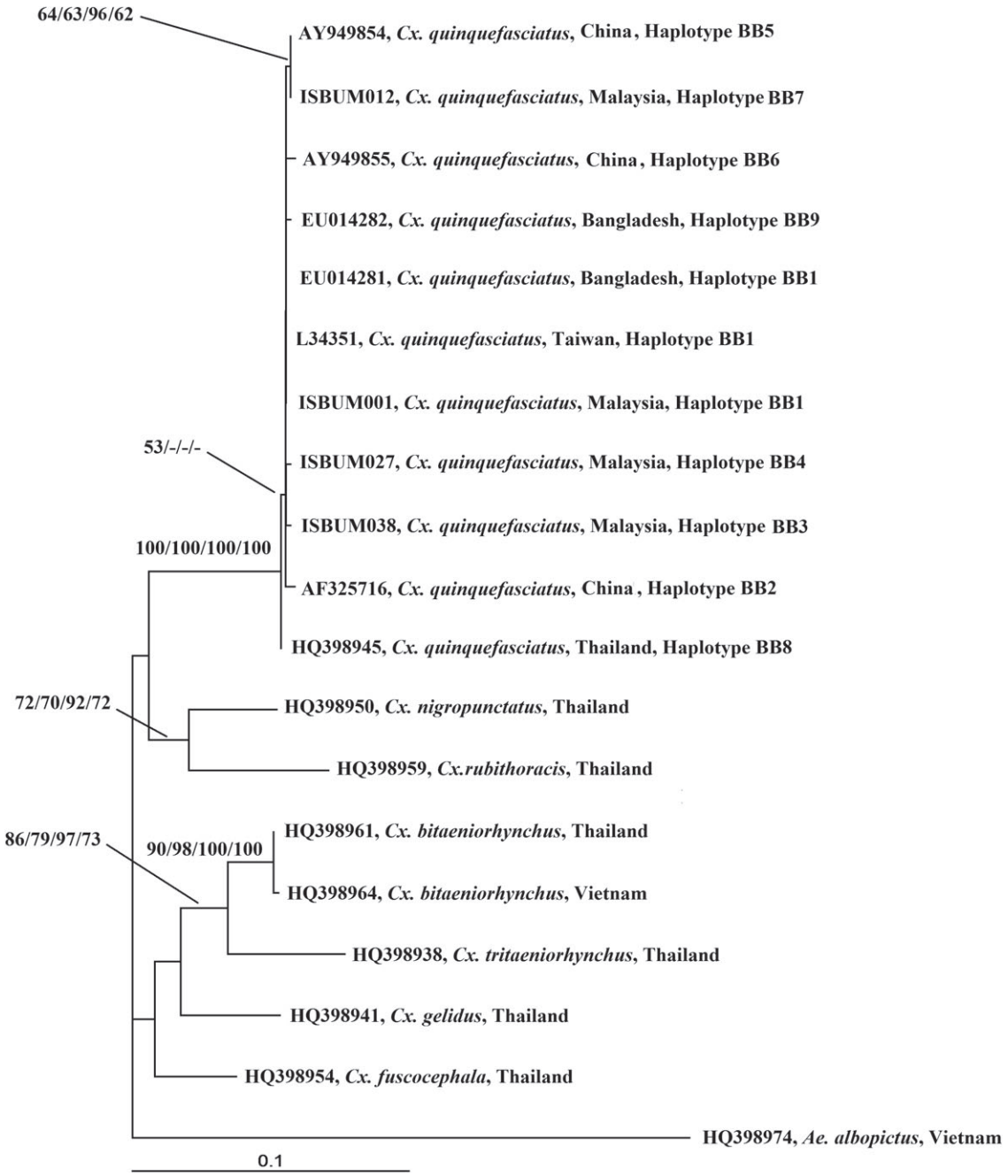

Fig. 4. Maximum likelihood phylogeny tree of *Culex* taxa based on COII sequences. Bootstrap [maximum likelihood (ML)/maximum parsimony (MP)/Bayesian inference (BI)/neighbour-joining (NJ)] values are shown at the branches. Bar indicates substitutions per site.

reported that Asian and East African *Cx. quinquefasciatus* were assigned to the same genetic cluster, suggesting that heavy human traffic across the Indian Ocean may account for the common genetic lineages (Fonseca *et al*., 2006).

A bottleneck effect may lead to a decline in genetic variability (in terms of average heterozygosity per locus) and cause incompetent adaptability of a species in a given population (Neil *et al*., 1975). In this context, Malaysian *Cx. quinquefasciatus* may have experienced a population bottleneck caused by the activities of vector-borne disease control programmes (i.e. larviciding, fogging, indoor residual spraying and physical elimination of breeding sources) conducted in Malaysia since 1967 and have consequently reduced in genetic variation. Similar conclusions were reported by Cartaxo *et al*. (2011), who found that lymphatic filariasis vector control programmes had reduced the genetic diversity of Brazilian *Cx. quinquefasciatus*, as evidenced by the monitoring of genetic diversity using microsatellites over a 3-year period.

In the context of insecticide resistance, several researchers have reported that insecticide-resistant mosquitoes exhibit a high degree of genetic variation, inferred from random amplified polymorphic DNA (Ocampo & Wesson, 2004; Sharma *et al*., 2009), 16S rRNA sequence (Sharma *et al*., 2010) and isozyme loci (Ayres *et al*., 2004). Although high genetic variation was observed in these previous studies, it does not exclude the possibility that the fixation of advantageous mutations associated with a hitchhiking effect may take place in other genome regions around a putative locus of insecticide resistance (Yan *et al*., 1998). According to a previous report by the present authors, Malaysian *Cx. quinquefasciatus* that were collected concurrently from the same localities developed a wide range of insecticide resistance towards dichlorodiphenyltrichloroethane (DDT), propoxur, malathion and permethrin (Low *et al*., 2013). It is important to point out that insecticide resistance has evolved in this mosquito species in Malaysia because this suggests that the evolution of insecticide resistance is associated with a hitchhiking effect and with consequently reduced levels of genetic variation.

It has also been reported that the bottleneck effect on genetic variation is more accentuated in mitochondrial than in nuclear loci as the genetic drift lacks the time to reduce variation at nuclear loci (Birungi & Munstermann, 2002). In future studies, it will be important to incorporate additional markers that target nuclear loci to further confirm the population genetic structure of *Cx. quinquefasciatus* in this region. There is now an urgent need to investigate the hitchhiking effect associated with insecticide resistance in this mosquito species and ultimately to establish complementary control strategies against these mosquito populations.

In addition to insecticide resistance, *Wolbachia*-infected *Cx. quinquefasciatus* populations have been proven to demonstrate a drastic reduction in mitochondrial variation (Rasgon *et al*., 2006; Behbahani, 2012). Given that *Wolbachia* infection is commonly found in Asian *Cx. quinquefasciatus* populations (Kittayapong *et al*., 2000; Ravikumar *et al*., 2011), it is suggested that the low mitochondrial diversity observed in the present study may also have derived from *Wolbachia* infection.

In the present study, the application of both the COI and COII genes revealed genetic lineages, dispersal patterns and hypothetical ancestral genotypes in *Cx. quinquefasciatus*. Further work, with increased numbers of specimens sourced from wider biogeographic areas in Malaysia, and the incorporation of additional markers that are more variable, will be beneficial in helping to unravel the presence of additional haplotypes.

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Current Susceptibility Status of Malaysian *Culex quinquefasciatus* (Diptera: Culicidae) Against DDT, Propoxur, Malathion, and Permethrin

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ABSTRACT A nationwide investigation was carried out to determine the current susceptibility status of *Culex quinquefasciatus* Say populations against four active ingredients representing four major insecticide classes: DDT, propoxur, malathion, and permethrin. Across 14 study sites, both larval and adult bioassays exhibited dissimilar trends in susceptibility. A correlation between propoxur and malathion resistance and between propoxur and permethrin resistance in larval bioassays was found. The results obtained from this study provide baseline information for vector control programs conducted by local authorities. The susceptibility status of this mosquito should be monitored from time to time to ensure the effectiveness of current vector control operations in Malaysia.

KEY WORDS *Culex quinquefasciatus*, WHO bioassay, insecticide susceptibility, cross-resistance, Malaysia

Culex quinquefasciatus Say (Diptera: Culicidae) is the most common Malaysian nuisance mosquito (Yap et al. 2000a, Low et al. 2012). It is also a potential vector of urban lymphatic filariasis caused by the nematode parasite, *Wuchereria bancrofti* in Malaysia (Vythilingam et al. 2005). Around the world, its significance as a vector of bancroftian filariasis (Samuel et al. 2004), West Nile virus (Sardelis et al. 2001, Pitzer et al. 2009), Saint Louis encephalitis virus (Jones et al. 2002), Ross River virus (Lindsay et al. 1993), and Japanese encephalitis virus (Nitatpattana et al. 2005) has been well documented.

Application of organochlorines, organophosphates, carbamates, and pyrethroids remain as the main control agents in vector control programs. However, the extensive use and over-reliance on insecticides have contributed to insecticide resistance development through the selection of certain genes (World Health Organization [WHO] 2006). In fact, insecticide resistance is not a new phenomenon and is an increasing problem worldwide. *Cx. quinquefasciatus* from different parts of the world have been reported to be resistant to various insecticide classes (Bisset et al. 1997, Chandre et al. 1997, Liu et al. 2004, Sathantriphop et al. 2006, Kasai et al. 2007, Pridgeon et al. 2008). Among the various mosquito control approaches, adulticiding with ultra low volume (ULV) fogging, thermal fogging, surface residual spray, or household insecticide products are specifically designed for the control of adult mosquitoes (Yap et al. 2000b). In many urban and suburban areas, larviciding is the most widely used method for the control of *Cx. quinquefasciatus* larvae, as high levels of adult organochlorine and organophosphate resistance have been reported (Chavasse and Yap 1997).

To date, no nationwide investigation of insecticide susceptibility status of wild *Cx. quinquefasciatus* has been reported in Malaysia. Over the years, the susceptibility status of wild *Cx. quinquefasciatus* against insecticides has been focused in the Klang Valley (Kuala Lumpur and Selangor), Pahang, and Penang (Reid 1955, Wharton 1958, Thomas 1962, Lee and Tadano 1994, Lee et al. 1997, Nazni et al. 2005) districts. There has been a dearth of information regarding the insecticide susceptibility status of wild *Cx. quinquefasciatus* in other districts of Malaysia. Hence, the present article is the first attempt to quantify the susceptibility status of wild *Cx. quinquefasciatus* against four active ingredients representing four major insecticide classes from each state of Malaysia, including East Malaysia. The findings of this study will be a timely reminder and an early warning to local authorities that systematic insecticide resistance management is essential for the improvement of current vector control operations in Malaysia.

Materials and Methods

Mosquito Strains. Mosquito larvae were collected from stagnant water at residential areas in each state

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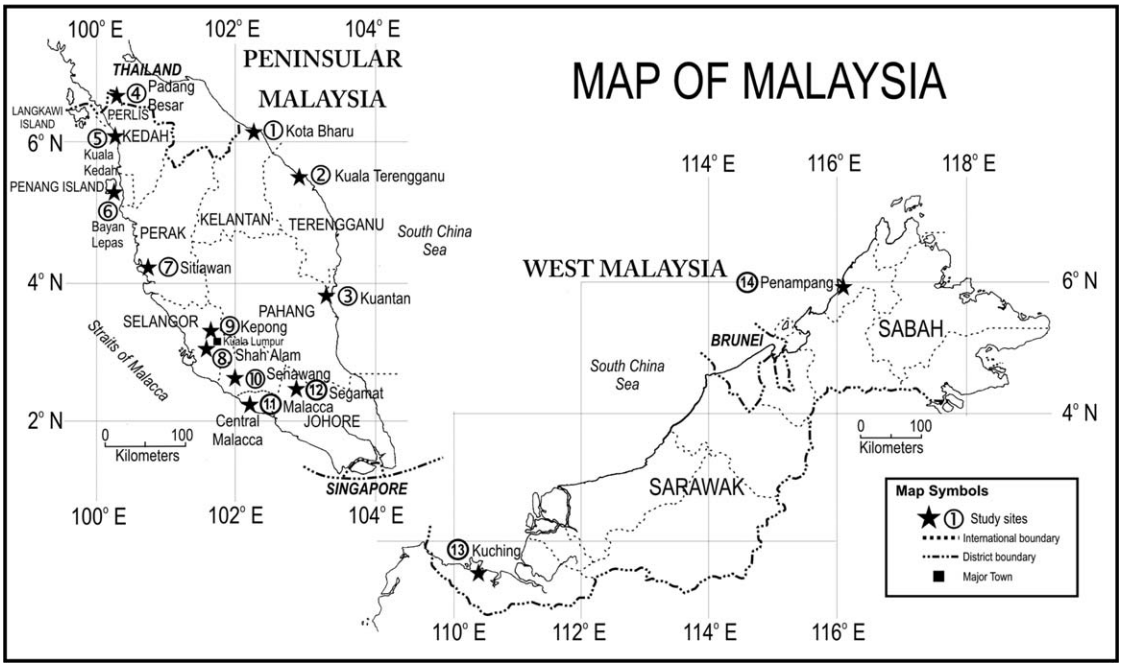

Fig. 1. Collection sites of *Culex quinquefasciatus* larvae in Malaysia.

of Malaysia (Fig. 1; Table 1), by using a previously described dipping method (Mendoza et al. 2008). Because there is no specific control program for *Culex* spp. mosquitoes in Malaysia, the selection criteria for these study sites were based on the frequent reports of dengue cases and fogging activities from these sites.

Field-collected larvae were transported to the laboratory and reared to adulthood. Larvae were provided with a fine mixture of mice chow, beef liver, and milk powder in the ratio of 2:1:1 by weight, while adults were provided with 10% sucrose solution. The emerging *Cx. quinquefasciatus* adults were identified

Table 1. Geographical description of mosquito collection sites across 14 states in Malaysia

Malaysia	Region	State	District	Study site	GPS coordinates	Landscape
Peninsular	East coast	Kelantan	Kota Bharu	Taman Guru	06° 05′49.43″ N, 102° 14′06.80″ E	Suburban
		Terengganu	Kuala Terengganu	Kg. Simpang Empat	05° 15′57.73″ N, 103° 10′49.90″ E	Rural
		Pahang	Kuantan	Taman Chenderawasih	03° 48′00.40″ N, 103° 18′02.20″ E	Suburban
	Northern	Perlis	Padang Besar	Taman Singgahsana	06° 39′11.00″ N, 100° 18′54.00″ E	Rural
		Kedah	Kuala Kedah	Taman Selat	06° 05′02.10″ N, 100° 18′07.70″ E	Suburban
		Penang	Bayan Lepas	Taman Bayan Baru	05° 19′46.51″ N, 100° 17′24.80″ E	Urban
		Perak	Sitiawan	Taman Bunga Ros	04° 12′42.21″ N, 100° 41′42.20″ E	Suburban
	Central	Selangor	Shah Alam	Section 17	03° 02′58.28″ N, 101° 30′16.40″ E	Urban
		Kuala Lumpur	Kepong	Kepong Baru	03° 12′18.23″ N, 101° 38′43.60″ E	Urban
	Southern	Negeri Sembilan	Senawang	Taman Marida	02° 41′52.40″ N, 101° 59′02.44″ E	Suburban
		Malacca	Central Malacca	Kg. Pengkalan Rama Pantai	02° 12′35.77″ N, 102° 15′02.52″ E	Rural
		Johore	Segamat	Segamat Baru	02° 29′56.50″ N, 102° 51′12.10″ E	Suburban
East Malaysia	West	Sarawak	Kuching	Taman Budaya	01° 33′10.11″ N, 110° 20′41.00″ E	Suburban
	East	Sabah	Penampang	Bundusan Villa	05° 56′22.34″ N, 116° 06′19.37″ E	Suburban

according to illustrated keys (Rattanarithikul et al. 2005) and cross-referenced with the voucher specimens from the laboratory. Three days after emergence, the *Cx. quinquefasciatus* female mosquitoes were blood-fed by using a BALB/c mouse. Three days after blood feeding, 300 ml capacity oviposition cups containing 200 ml deionized water were introduced into mosquito cages (33 × 33 × 33 cm). The hatched larvae were designated as first generation (F1), which were subsequently used for larval susceptibility bioassays while adults from F1 reared larvae were used for adult susceptibility bioassays. For comparison purposes, a laboratory reference strain of *Cx. quinquefasciatus* from the Institute for Medical Research, Kuala Lumpur, which has been cultured under insecticide-free conditions for 117 generations was used.

Insecticides. Four active ingredients representing four major insecticide classes used in both larval and adult susceptibility tests. These included an organochlorine (DDT), a carbamate (propoxur), an organophosphate (malathion), and a pyrethroid (permethrin). DDT 4.0%, propoxur 16%, malathion 8%, and permethrin 0.5% in solution form and the Whatman No. 1 filter papers (12 × 15 cm) that were impregnated with 2 ml of the diagnostic concentrations of DDT 4.0%, propoxur 0.1%, malathion 5.0%, and permethrin 0.25%, respectively, were purchased from WHOPES Collaborating Centre in Universiti Sains Malaysia, Penang.

Larval Susceptibility Test. This test was conducted according to the WHO (1981a) larval susceptibility bioassay procedure. Stock solutions of each insecticide were made up in ethanol and further diluted with the desired concentrations. Briefly, the bioassay was conducted in 300 ml disposable paper cups. The prepared stock solution of insecticide was added into 150 ml deionized water. Five concentrations and three containers (25 late third or early fourth instar larvae per replicate) per concentration were performed with each insecticide, for example, DDT (0.500–4.400 mg/L), propoxur (0.030–1.400 mg/L), malathion (0.020–1.900 mg/L), and permethrin (0.030–1.800 mg/L). After introducing the larvae into paper cups, water was added to make the final volume to 250 ml. The control (untreated) was set up by adding 1 ml of ethanol into the paper cups containing 249 ml deionized water. Larval mortality was recorded after 24 h of continuous exposure. Moribund larvae were counted as dead.

Adult Susceptibility Test. This test was conducted according to the WHO (1981b) adult susceptibility bioassay procedure, with minor modifications. A batch of 15 sucrose-fed, 3- to 5-d-old female mosquitoes was exposed to the diagnostic WHO-impregnated papers and the test was repeated three times. Briefly, the mosquitoes were removed from the cage by using a plastic aspirator tube and transferred into WHO exposure tubes (125 mm in length, 44 mm in diameter). Test tubes were covered with black cloth to ensure that mosquitoes would rest on the impregnated paper. For the determination of KT_{50} (50% knockdown time) value, the number of mosquitoes knocked down was recorded every minute (Lee et al. 1997, Nazni et al. 2009) for DDT, propoxur, malathion, and permethrin during exposure periods of 4, 2, 1, and 3 h, respectively. Mosquitoes that survived the cumulative exposure period were transferred to WHO holding tubes to allow an observation of posttreatment effect. Controls were exposed to nontreated paper. Cotton pads soaked in 10% sugar solution were provided during the 24 h postexposure period. Mortality was recorded 24 h after the initial exposure period.

Statistical Analysis. Larval bioassay data within the range of 5–95% were subjected to probit analysis (Finney 1971) using a computerized program, PROBIT (National Center for Scientific Research, France) developed by Raymond (1993). Based on the LC_{50} obtained from larval bioassays, resistance ratios (RR) were calculated by dividing values for the resistant strain by those of the susceptible strain (Brown and Pal 1971). Calculated RR values >10 are indicative of high resistance, 5–10 are indicative of medium resistance, and <5 are indicative of low resistance (Mazarri and Georghiou 1995). The associations between the RR values in larval bioassays were accessed by Spearman rank-order correlation, for the determination of cross-resistance, as described by Bisset et al. (1997).

With regards to adult bioassays, a specific time for each chemical's knockdown analysis was performed based on the KD of the reference strain. To best describe the susceptibility status, knockdown evaluation of DDT, propoxur, malathion, and permethrin were performed at 80, 50, 70, and 50% of the total exposure time, respectively. The percentage mortality at 24 h posttreatment was used to determine susceptibility status: 98–100% mortality indicates susceptibility, 80–97% mortality suggests the possibility of resistance that needs to be further confirmed, and <80% mortality suggests resistance (WHO 2009). Abbott's formula (Abbott 1925) was applied to correct percentage mortality if control mortality was >5%. Comparative measure of knockdown and mortality between the study sites was performed by one-way analysis of variance (ANOVA) (dependent variable = knockdown/mortality; factor = study site). Tukey's test was used to separate means in significant ANOVAs, $P < 0.05$. Spearman rank-order correlation between the mortality percentages in adult bioassays were performed for the determination of cross-resistance (Bisset et al. 1997).

Results

The susceptibility status of *Cx. quinquefasciatus* against DDT, propoxur, malathion, and permethrin in larval and adult stages are presented in Tables 2 and 3, respectively. In each insecticide tested, both larval and adult bioassays exhibited dissimilar trends in susceptibility across all study sites. Various insecticide susceptibility levels (susceptible, low to high resistance) in both larval and adult bioassays were demonstrated from different localities in Malaysia.

Larval bioassays demonstrated various resistance ratios, ranging from 0.66 to 3.83, 0.38–2.93, 0.36–13.88,

Table 2. DDT, propoxur, malathion, and permethrin susceptibility for several Malaysian *Culex quinquefasciatus* larval strains

Strain	DDT		Propoxur		Malathion		Permethrin	
	LC_{50} (mg/L) 95% (CL)	RR_{50}	LC_{50} (mg/L) 95% (CL)	RR_{50}	LC_{50} (mg/L) 95% (CL)	RR_{50}	LC_{50} (mg/L) 95% (CL)	RR_{50}
Reference	1.009 (1.057–1.143)	—	0.242 (0.234–0.251)	—	0.124 (0.114–0.135)	—	0.211 (0.167–0.196)	—
Kelantan	1.226 (1.134–1.137)	1.12[a]	0.416 (0.382–0.454)	1.72[a]	0.045 (0.041–0.049)	0.36[b]	0.714 (0.564–0.895)	3.38[a]
Terengganu	0.968 (0.846–1.122)	0.88	0.092 (0.069–0.115)	0.38[b]	0.057 (0.046–0.070)	0.46[b]	0.143 (0.119–0.169)	0.68
Pahang	2.758 (2.709–2.802)	2.51[a]	0.168 (0.146–0.189)	0.69[b]	0.168 (0.146–0.189)	1.35[a]	0.177 (0.152–0.203)	0.84
Perlis	3.440 (3.355–3.535)	3.13[a]	0.146 (0.116–0.177)	0.60[b]	0.055 (0.051–0.060)	0.44[b]	0.049 (0.045–0.055)	0.23[b]
Kedah	3.508 (3.418–3.589)	3.19[a]	0.489 (0.442–0.542)	2.02[a]	0.864 (0.812–0.913)	6.97[a]	0.187 (0.162–0.216)	0.89
Penang	1.325 (1.230–1.417)	1.21[a]	0.708 (0.609–0.809)	2.93[a]	1.271 (1.216–1.322)	10.25[a]	0.375 (0.326–0.429)	1.78[a]
Perak	0.725 (0.618–0.898)	0.66[b]	0.175 (0.153–0.196)	0.72[b]	0.541 (0.514–0.568)	4.36[a]	0.056 (0.051–0.063)	0.27[b]
Selangor	3.155 (3.043–3.284)	2.87[a]	0.656 (0.580–0.725)	2.71[a]	1.650 (1.592–1.714)	13.31[a]	0.803 (0.706–0.910)	3.81[a]
Kuala Lumpur	3.067 (2.991–3.175)	2.79[a]	0.623 (0.569–0.687)	2.57[a]	1.721 (1.651–1.806)	13.88[a]	0.472 (0.426–0.518)	2.24[a]
Negeri Sembilan	4.205 (4.160–4.251)	3.83[a]	0.456 (0.390–0.571)	1.88[a]	1.247 (1.203–1.300)	10.06[a]	0.504 (0.457–0.558)	2.39[a]
Malacca	3.695 (3.656–3.735)	3.36[a]	0.463 (0.413–0.514)	1.91[a]	1.309 (1.240–1.372)	10.57[a]	0.430 (0.401–0.463)	2.04[a]
Johore	3.705 (3.664–3.747)	3.37[a]	0.607 (0.547–0.675)	2.51[a]	1.009 (0.964–1.059)	8.14[a]	0.389 (0.337–0.451)	1.84[a]
Sarawak	2.684 (2.587–2.781)	2.44[a]	0.471 (0.426–0.517)	1.95[a]	0.351 (0.306–0.396)	2.83[a]	0.428 (0.406–0.449)	2.03[a]
Sabah	1.064 (0.974–1.159)	0.97	0.384 (0.332–0.446)	1.59[a]	0.700 (0.647–0.759)	5.65[a]	0.137 (0.108–0.174)	0.65

Reference strain was obtained from Institute for Medical Research, Kuala Lumpur, Malaysia. Mosquito larvae collected from the field were reared to F1.

[a] CL does not overlap with the reference strain and significantly different from the reference strain.

[b] Strain with a significant lower resistance ratio.

and 0.23–3.81 fold for DDT, propoxur, malathion, and permethrin, respectively. It is important to point out that the *Cx. quinquefasciatus* larvae from Terengganu were susceptible to all four insecticides, having resistance ratios <1. The *Cx. quinquefasciatus* larvae from Kuala Lumpur, Selangor, Malacca, Penang, and Negeri Sembilan were most resistant to malathion by exhibiting resistance ratios >10. In addition, Spearman rank-order correlation indicated a significant correlation between resistance ratios of propoxur and malathion ($r = 0.780$; $P = 0.001$) and between resistance ratios of propoxur and permethrin ($r = 0.613$; $P = 0.020$) in larval bioassays (Fig. 2), while no correlation was found with other insecticides in either larval or adult bioassays.

In adult bioassays, DDT resistance was expressed most frequently among the four insecticides evaluated, as 0% knockdown was recorded at 80% of the total exposure time from 12 out of 14 of the populations. Meanwhile, 0% knockdown was detected from eight out of 14 and five out of 14 of the populations using propoxur at 50% of the total exposure time and malathion at 70% of the total exposure time, respectively. A wide spectrum of knockdown was detected with permethrin evaluated at 50% of the total exposure time across all study sites.

Across all study sites, DDT and propoxur exhibited <40% and 70% mortality, respectively, whereas complete mortality was observed in malathion and permethrin from a few populations (Table 3). The results indicated that *Cx. quinquefasciatus* was most susceptible to permethrin. One-way ANOVA revealed that the susceptibility status of *Cx. quinquefasciatus* adults to various insecticides were significantly different across all study sites ($F = 48.16$; df = 13, 28; $P < 0.0001$).

With respect to both larval and adult bioassays that showed similar trends in susceptibility, both *Cx. quinquefasciatus* larvae and adults from Kelantan, Terengganu, and Perlis were susceptible to malathion. *Cx. quinquefasciatus* from Kuala Lumpur, Selangor, Malacca, Penang, and Negeri Sembilan were also apparently resistant to malathion with resistance ratios >10 in larval bioassays and low knockdown rate observed in adult bioassays. Meanwhile, inconsistency of mosquito susceptibility in both larval and adult stages from other districts against other insecticides was recorded.

Discussion

In the current study, mosquitoes from collection sites across Malaysia evaluated in both larval and adult bioassays exhibited dissimilar trends in susceptibility against the four insecticides tested. The occurrence of these incidences might be because of the differences between the insecticide resistance gene expression in larval and adult stages. A number of studies have indicated that insecticide resistance is more accentuated in the larval stage (Nazni et al. 2005; Selvi et al. 2006, 2007; Li and Liu 2010), while a lack of expression was observed in the adult stage (Huchard et al. 2006). However, higher levels of insecticide resistance in the adult stage also have been observed (Chavasse and Yap 1997). Cross-stage resistance has been reported because of the overlapping of certain mechanisms in response to insecticide pressure (Li and Liu 2010).

Generally, among the four insecticides tested in this study, Malaysian *Cx. quinquefasciatus* larvae were most resistant to malathion. Several conventional organophosphates have been introduced as larvicides for the control of mosquito larvae in Malaysia (Yap et al. 2000b). The occurrence of high level malathion resistance in the larval stage may be because of the over-usage of organophosphorus insecticides, resulting in the selection of one or more genes within exposed mosquitoes because of the use of compounds that share the same mode of action (Liu et al. 2004,

Table 3. Knockdown and mortality of larval-reared Malaysian *Culex quinquefasciatus* adults using a WHOPES treated filter paper assay

Strain	Knockdown (%)				Mortality (%)			
	DDT 4.0%	Propoxur 0.1%	Malathion 5.0%	Permethrin 0.25%	DDT 4.0%	Propoxur 0.1%	Malathion 5.0%	Permethrin 0.25%
Reference	4.44 ± 4.44	75.55 ± 9.69	51.11 ± 2.22	86.67 ± 3.85	43.34 ± 2.72	100.00 ± 0.00	100 ± 0.00	100.00 ± 0.00
Kelantan	0.00 ± 0.00a	0.00 ± 0.00a	6.67 ± 0.00ab	20.00 ± 6.67ab	^{R}24.44 ± 4.44cde	^{R}3.34 ± 3.34a	^{M}96.67 ± 3.34ef	^{R}43.33 ± 10.00ab
Terengganu	0.00 ± 0.00a	50.00 ± 10.00c	40.00 ± 0.00e	35.00 ± 5.00abc	^{R}25.00 ± 5.00cde	^{R}55.00 ± 5.00cd	^{S}100.00 ± 0.00f	^{M}95.00 ± 5.00de
Pahang	0.00 ± 0.00a	0.00 ± 0.00a	33.33 ± 3.33de	26.67 ± 10.19abcd	^{R}13.33 ± 3.33abc	^{R}20.00 ± 5.77ab	^{S}100.00 ± 0.00f	^{R}36.67 ± 3.33a
Perlis	0.00 ± 0.00a	22.22 ± 4.45bc	26.67 ± 3.85cde	24.45 ± 2.22ab	^{R}2.22 ± 2.22ab	^{R}68.89 ± 5.88d	^{R}75.55 ± 2.22def	^{R}71.11 ± 2.22cd
Kedah	0.00 ± 0.00a	0.00 ± 0.00a	13.33 ± 3.85abc	22.22 ± 5.88ab	^{R}4.45 ± 2.22ab	^{R}6.67 ± 3.85a	^{R}71.11 ± 5.88de	^{R}37.78 ± 2.22ab
Penang	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	20.00 ± 0.00abc	^{R}35.56 ± 5.88de	^{R}37.78 ± 2.22bc	^{R}35.56 ± 5.88bc	^{M}76.67 ± 10.00cde
Perak	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	36.67 ± 3.34abcd	^{R}20.00 ± 0.00bcd	^{R}6.67 ± 6.67a	^{R}10.00 ± 3.33ab	^{M}83.33 ± 10.00cde
Selangor	0.00 ± 0.00a	8.89 ± 2.22ab	0.00 ± 0.00a	35.56 ± 4.44abd	^{R}6.67 ± 3.85abc	^{R}55.55 ± 2.22cd	^{R}0.00 ± 0.00a	^{R}62.22 ± 4.45bc
Kuala Lumpur	0.00 ± 0.00a	4.45 ± 2.22a	0.00 ± 0.00a	13.33 ± 3.85a	^{R}17.78 ± 2.22abcd	^{R}33.33 ± 3.85bc	^{R}11.11 ± 5.88ab	^{R}77.78 ± 2.22cde
Negeri Sembilan	2.22 ± 2.22a	0.00 ± 0.00a	0.00 ± 0.00a	5.00 ± 5.00ab	^{R}20.00 ± 0.00bcd	^{R}10.00 ± 5.77a	^{R}6.67 ± 6.67ab	^{S}100.00 ± 0.00e
Malacca	0.00 ± 0.00a	0.00 ± 0.00a	2.33 ± 2.22a	35.55 ± 9.69abcd	^{R}11.11 ± 2.22abc	^{R}15.55 ± 2.22ab	^{R}4.44 ± 4.44ab	^{M}82.22 ± 5.88cde
Johore	0.00 ± 0.00a	0.00 ± 0.00a	2.22 ± 2.22a	70.00 ± 3.33cd	^{R}2.22 ± 2.22ab	^{R}22.22 ± 2.22abc	^{R}31.11 ± 4.44bc	^{S}100.00 ± 0.00e
Sarawak	0.00 ± 0.00a	13.33 ± 3.85ab	20.00 ± 6.67cde	75.56 ± 5.88d	^{R}0.00 ± 0.00a	^{R}53.33 ± 3.85d	^{R}62.22 ± 5.88d	^{S}100.00 ± 0.00e
Sabah	28.89 ± 2.22b	35.56 ± 4.44c	24.44 ± 4.44bcd	56.67 ± 3.34bcd	^{R}40.00 ± 3.85e	^{R}62.22 ± 4.45d	^{R}55.55 ± 2.22cd	^{M}93.33 ± 0.00d
One way ANOVA	F = 74.53 df = 13, 28 P < 0.0001	F = 23.13 df = 13, 28 P < 0.0001	F = 18.07 df = 13, 28 P < 0.0001	F = 1026.00 df = 13, 28 P < 0.0001	F = 13.40 df = 13, 28 P < 0.0001	F = 28.44 df = 13, 28 P < 0.0001	F = 48.16 df = 13, 28 P < 0.0001	F = 24.60 df = 13, 28 P < 0.0001

Knockdown evaluation performed at 80, 50, 70, and 50% of the total exposure time of DDT (4 h), propoxur (2 h), malathion (1 h), and permethrin (3 h), respectively. Mortality percentage recorded 24 h after the initial exposure period. Means followed by a different letter were significantly different, $P < 0.05$, Tukey's test.

R = resistant, S = susceptible, M = moderate resistant as determined by WHO (2009).

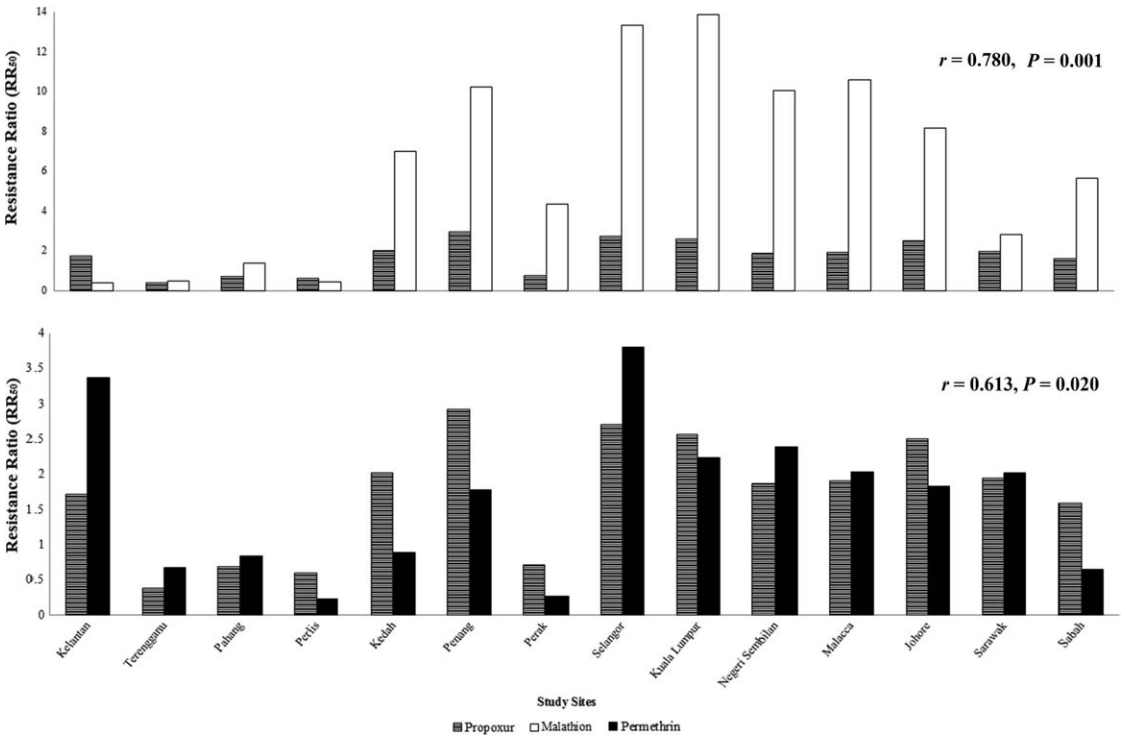

Fig. 2. Spearman rank-order correlation between resistance ratio of *Culex quinquefasciatus* larvae against propoxur, malathion, and permethrin.

Selvi et al. 2005). In the current study, malathion resistance was more accentuated in larval stage, as higher levels of malathion resistance was demonstrated in larvae, compared with adults. Likewise, a previous study also reported higher levels of malathion resistance in the larval stage (Selvi et al. 2005). However, manifold expression of organophosphate resistance in *Cx. quinquefasciatus* adults has been frequently reported (Chavasse and Yap 1997). Moreover, the increasing trend of esterase activities from the egg to adult stage has been observed in malathion resistant strains (Selvi et al. 2007). Hence, biochemical test should be conducted to identify the malathion resistance mechanism in Malaysian *Cx. quinquefasciatus* populations. Statistical analysis indicated that there was a significant correlation between propoxur and permethrin resistance and between propoxur and malathion resistance, suggesting the presence of cross-resistance. Although cross-resistance between propoxur and permethrin in this species has been observed previously (Sathantriphop et al. 2006), the actual mechanism(s) that caused this phenomenon remain questionable. However, cross-resistance between pyrethroid and carbamate in *Anopheles funestus* Giles has been reported and suggested that elevated levels of mixed function oxidases conferred cross-resistance in both classes of insecticides (Brooke et al. 2001, Cuamba et al. 2010). Conversely, cross-resistance between organophosphates and carbamates in *Cx. quinquefasciatus* has been documented frequently (Bisset et al. 1990, Chandre et al. 1997, Liu et al. 2004, Selvi et al. 2005). In addition to identifying the resistance gene that conferred organophosphate and carbamate resistance, acetylcholinesterase (AChE) insensitivity has been confirmed through molecular characterization (Cui et al. 2006, Alout et al. 2007).

Adult bioassays indicated that Malaysian *Cx. quinquefasciatus* were highly resistant to DDT. The laboratory reference strain also exhibited low susceptibility to DDT (% mortality = 43.34). Several mosquito species have expressed and maintained DDT resistance. Nazni et al. (2005) documented high DDT KT_{50} values in a laboratory reference strain and suggested that DDT was the least effective insecticide among all tested insecticides. Although DDT applications as indoor residual spraying was stopped in Malaysia in 1998, the resistance phenotype still remains in this mosquito population, suggesting that DDT would be ineffective. Similarly, a DDT resistance phenotype remained in a laboratory reference strain of *Aedes aegypti* (L.) although this strain has been cultured under insecticide-free conditions for 1,014 generations (Nazni et al. 2009). Furthermore, high levels of DDT resistance persist in *Cx. pipiens* populations from Egypt, although this insecticide has not been used since the 1970s (Zayed et al. 2006).

Among the four insecticides tested in this study, *Cx quinquefasciatus* was most susceptible to permethrin. However, low permethrin resistance was detected in these populations. Pyrethroids are the most important

class of insecticide with major usage in public health and household insecticide products (Yap et al. 2000b). The extensive usage of this insecticide may lead to pyrethroid resistance development in this species. In 1996, the introduction of permethrin fogging activities contributed to permethrin resistance development in *Cx. quinquefasciatus* (Nazni et al. 1998). Moreover, as this species prefers to rest indoors (Tham 2000), it is more likely to be exposed to pyrethroid-based household insecticide products. This is further supported by Yap et al. (1995), where *Cx. quinquefasciatus* was most tolerant to household insecticide products containing pyrethroids as the active ingredient. Several formulations of household insecticide products such as coils, mats, liquid vaporizer, and aerosol have been introduced widely in Malaysian markets. The mentioned formulations contained the active ingredient of d-allethrin, d-*trans* allethrin, transfluthrin, prallethrin, s-bioallethrin, deltamethrin, d-phenothrin, permethrin, and tetramethrin (Yap et al. 2000b). It is suggested that the over-reliance of these pyrethroid-based household insecticide products conferred the low permethrin resistance detected in this study.

The current findings indicated that *Cx. quinquefasciatus* from Selangor and Kuala Lumpur exhibited a similar trend of resistance against malathion. In recent years, a similar study showed that *Cx. quinquefasciatus* larvae and adults from Kuala Lumpur were highly resistant to malathion (Nazni et al. 2005). To date, numerous dengue and chikungunya cases from the areas of Kuala Lumpur and Selangor have been frequently reported to the Ministry of Health, Malaysia. To control the spread of these mosquito-borne pathogens, fogging activities have been frequently carried out in these endemic areas. As a consequence, *Cx. quinquefasciatus* may have developed insecticide resistance through this selection pressure. Because of the high frequency of fogging activities, these areas also were targeted for insecticide resistance studies by Chen et al. (2005a, b) and Nazni et al. (2005), which provides a strong comparison to the current study.

Several resistance reports of Malaysian wild *Cx. quinquefasciatus* against DDT (Reid, 1955, Thomas 1962, Nazni et al. 2005), propoxur (Nazni et al. 2005), malathion (Lee et al. 1997, Nazni et al. 2005), and permethrin (Lee et al. 1997, Nazni et al. 2005) have been reported previously in a few states in Malaysia, although these previous results could not be compared directly because of different handling methods and procedures. In the current study, insecticide susceptibility status of Malaysian *Cx. quinquefasciatus* larvae and adults has been demonstrated throughout the country, indicating that different localities should be targeted with different chemicals. The findings of the current study may assist local authorities by providing an updated susceptibility baseline and data to be used for choosing application rates and insecticides for vector control operations. With respect to the knockdown rates observed in the adult bioassays, certain populations displayed 0% knockdown making it difficult to choose an appropriate application rate for an adulticiding program. Therefore, it is important to incorporate another resistance monitoring method, such as topical application to confirm the susceptibility status of this mosquito species in Malaysia.

Although the resistance ratio reported from most of the study sites could be considered low, it nevertheless indicates that resistance is developing and preventive measures should be considered proactively. However, insecticide resistance was detected in several populations, thereby allowing for biochemical and molecular studies to characterize the mechanism involved in *Cx. quinquefasciatus* resistance.

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First molecular genotyping of voltage gated sodium channel alleles in *Culex quinquefasciatus* populations in Malaysia

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ABSTRACT

A nationwide investigation was performed to detect the presence of 1014 mutation(s) in voltage gated sodium channel (*kdr*) gene of *Culex quinquefasciatus* from 14 residential areas across 13 states and a federal territory in Malaysia. Molecular genotyping of *kdr* mutation was performed via a modified three tubes allele-specific-polymerase chain reaction (AS-PCR) and direct sequencing of *kdr* gene. Based on the results of AS-PCR, homozygous susceptible (SS) genotype was found in nine out of 14 populations with 38 individuals from a total sample size of 140. Heterozygous (RS) genotype was most predominant (99 individuals) and distributed across all study sites. Homozygous resistance (RR) genotype was detected in Perak (one individual) and Selangor (two individuals). The resistance *kdr* allele frequencies ranged from 0.1 to 0.55, with the highest being detected in *Cx. quinquefasciatus* population from Selangor. This study has documented the first field-evolved instance of 1014F mutation in Malaysian mosquitoes and the findings of this study could be utilized in the implementation of strategic measures in vector control programs in Malaysia.

1. Introduction

Globally, the evolution of multiple or cross insecticide resistance in medically and agriculturally important insect pests is a major limiting factor in the advancement of vector/pest control management [1,2]. In the last few decades, organochlorine insecticides (i.e., DDT) have been heavily used in pest control programs [2]. However, while the ultimate or progressively evolving DDT resistance in insect pests were documented, in recent decades, pyrethroid-based insecticides have been introduced as alternatives to DDT [3]. Both pyrethroids and DDT attack the voltage-gated sodium channel of insects leading to the development of knockdown resistance when there is an excessive use of either class of insecticide [4].

Knockdown resistance is not a new phenomenon and is an increasing problem in every part of the world. Knockdown resistance has been the subject of research interest among researchers for more than 50 years and intensive research efforts have unraveled the underlying mechanisms that conferred knockdown resistance at a molecular level [5]. Over the years, knockdown resistance have been extensively reported in a number of insect pests (i.e., mosquitoes, cockroaches, ticks, lice, house flies, horn flies, fruit flies, white flies, aphids, beetles, and moths), as reviewed by Soderlund and Knipple [5], Hemingway et al. [4] and Liu et al. [6].

In Malaysia, mosquitoes are important insect vectors/pests and the application of insecticides remains the main method of control in mosquito control programs [7]. Specifically, *Culex quinquefasciatus* Say is the most abundant Malaysian pest mosquito [8,9]. Insecticide resistance towards DDT and pyrethroids in Malaysian *Cx. quinquefasciatus* have been frequently reported [10–15]. However, in Malaysia, research efforts have mainly focused on the biochemical characterization of enzyme-based metabolic mechanisms [12,14]. Indeed, there is a lack of evidence of insecticide resistance conferred by mutations in the voltage gated sodium channel in Malaysian mosquitoes as well as other insect species in Malaysia.

According to our previous report [15], both WHO larval and adult bioassays revealed that Malaysian *Cx. quinquefasciatus* has developed a wide spectrum of insecticide resistance towards DDT and permethrin. In particular, DDT resistance was expressed most frequently, as 0% knockdown was recorded from 12 out of 14 of the populations [15]. In this context, it is of paramount importance to investigate the knockdown resistance at a molecular level and thereby attempting to determine the prevalence of the *kdr* mutation in *Cx. quinquefasciatus* populations from all states and a federal territory in Malaysia.

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2. Materials and methods

2.1. Ethical notes

This research was regulated by the Medical Review & Ethics Committee (MREC), Ministry of Health Malaysia. No specific permits were required for this study. This study also did not involve endangered or protected species.

2.2. Mosquito strains

The selection criteria for the study sites were based on the frequency of reports of dengue cases and fogging activities, as dengue is the most prevalent mosquito-borne viral disease in Malaysia. Specific mosquito control programs mainly target *Aedes* and not *Culex* mosquitoes. However, widespread fogging against dengue vectors would also exert selective pressure on *Cx. quinquefasciatus*, as the fogged insecticides, mainly pyrethroids would inadvertently contaminate *Cx. quinquefasciatus* breeding grounds such as polluted drains.

A nationwide *Culex* larval survey was carried out at 14 dengue endemic residential areas across 11 states and a federal territory (i.e., Kuala Lumpur) in Peninsular Malaysia and two states in East Malaysia (Fig. 1). Details of the studied study sites and sample collections have been described elsewhere [15]. Field-collected larvae were transported to the laboratory and reared to adulthood for identification using taxonomic keys [16]. In the present study, a total of 140 adults *Cx. quinquefasciatus* with 10 individual mosquitoes representing each of the 14 study sites were randomly selected.

2.3. DNA extraction

Prior to DNA extraction, abdomens were dissected out of the mosquito samples to avoid contamination. DNA was extracted from each specimen using i-genomic CTB DNA Extraction Mini Kit (iNtRON Biotechnology Inc., Kyungki-Do, South Korea). All isolation steps were performed according to manufacturer instructions.

2.4. Detection of kdr mutation by allele-specific (AS)-PCR method

A modified three tubes AS-PCR method [17–18] was performed to detect the presence of 1014F and 1014S alleles. Three separate PCR reactions were conducted by using the mixture of CD1 primer, 5′-AAC TTC ACC GAC TTC ATG CAC-3′ and CD2 primer, 5′-CAA GGC TAA GAA AAG GTT AAG AAC-3′ with CD3 specific primer, 5′-CCA CCG TAG TGA TAG GAA ATT TA-3′ for the TTA (Leu) detection, CD4 specific primer, 5′-CCA CCG TAG TGA TAG GAA ATT TT-3′ for the TTT (Phe) detection or CD5 specific primer, 5′-CCA CCG TAG TGA TAG GAA ATT C-3′ for the TCA (Ser) detection. The ratio of the primer mixture was CD1:CD2:CD3/4/5 = 3:10:7. The control product of 490-bp was amplified from primers CD1 and CD2 while the 370-bp fragment was the *kdr*-specific allele from primers CD3, CD4 and CD5.

The amplification of sodium channel region was performed in a final volume of 25 μL containing 25–50 ng genomic DNA of mosquito, 12 μL of ExPrime Taq Master Mix (GENETBIO Inc., Daejeon, South Korea) and 2 μL of primer mixture. PCR was carried out using a Bio-rad MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The PCR conditions included an initial denaturation of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s (denaturation), 60 °C for 30 s (annealing), 72 °C for 45 s (extension) and a final extension at 72 °C for 10 min [18].

The amplified fragments were electrophoresed on 2% agarose gel pre-stained with SYBR Safe (Invitrogen, Carlsbad, CA) in TAE buffer.

2.5. Detection of kdr mutation by sequencing method

A subset of 40 individual samples from the 140 samples tested was screened for *kdr* mutation by direct sequencing. We designed new primers based on our cloned sequences (KC189872 and KC189873): JKDR_F, forward primer, 5′-GGA TCG AAT CCA TGT GGG ACT-3′ and JKDR_R, reverse primer, 5′-TGC ACC TTT AGG TGT GGA CCT TC-3′.

The amplification of sodium channel region was performed in a final volume of 50 μL containing 5 μL 10× buffer, 2.5 mM of each dNTP, 10 pmol of each forward and reverse primer, 1.5 U *Taq* polymerase (iNtRON Biotechnology Inc., Kyungki-Do, South Korea), and 25–50 ng genomic DNA of mosquito. PCR was carried out using Bio-Rad MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The PCR conditions included an initial denaturation of 94 °C for 5 min, followed by 40 cycles of 94 °C for 45 s (denaturation), 59 °C for 45 s (annealing), 72 °C for 45 s (extension) and a final extension at 72 °C for 5 min.

The amplified fragments (~285 bp) were electrophoresed on 2% agarose gel pre-stained with SYBR Safe™ (Invitrogen, Carlsbad, CA) in TAE buffer. The PCR products were purified with MEGAquick-spin PCR & Agarose Gel DNA Extraction System (iNtRON Biotechnology Inc., Kyungki-Do, South Korea).

The purified PCR products were sent to a commercial company for DNA sequencing in both directions. Samples were sequenced using BigDyeH Terminator v3.1 Sequencing Cycle Kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI PRISM 377 genetic analyzer (Applied Biosystems, Foster City, CA).

Sequencing data were analyzed and edited using ChromasPro 1.5 (Technelysium Pty Ltd., Qld, Australia) and BioEdit 7.0.9.0. [19]. The sodium channel sequences were preliminarily aligned using the CLUSTAL X program [20] and subsequently aligned manually. Representative sequences of the sodium channel gene of *Cx. quinquefasciatus* in this study were deposited in GenBank under the accession numbers KC189872–KC189889.

2.6. Statistical analysis

The frequencies of *kdr* allele were determined by Hardy–Weinberg Equilibrium, using GenePOP (ver 3.4) software [21].

3. Results

The AS-PCR method demonstrated the presence of the classical 1014F mutation in all of the wild populations of *Cx. quinquefasciatus* tested, while the 1014S mutation was not detected across all study sites in Malaysia (Fig. 1 and Table 1). Overall, the SS genotype was found in a majority of the study sites (nine out of 14) with 38 individuals from a total sample size of 140. The RS genotype was detected across all study sites and was most predominant with 99 individuals from a total sample size of 140. Of 14 populations, two populations (i.e., Perak and Selangor) indicated the presence RR genotype with three individuals. It is of interest that the SS genotype was not detected in five populations (i.e., Kuala Lumpur, Malacca, Negeri Sembilan, Penang and Perlis).

The genotype frequencies at *kdr* locus from seven populations (i.e., Johore, Kedah, Kelantan, Sabah, Sarawak, Selangor and Terengganu) conformed to the Hardy–Weinberg expectations at the 95% confidence level ($P > 0.05$). Inversely, the genotype frequencies at *kdr* locus from another seven populations (i.e., Kuala Lumpur, Malacca, Negeri Sembilan, Pahang, Penang, Perak and Perlis) differed

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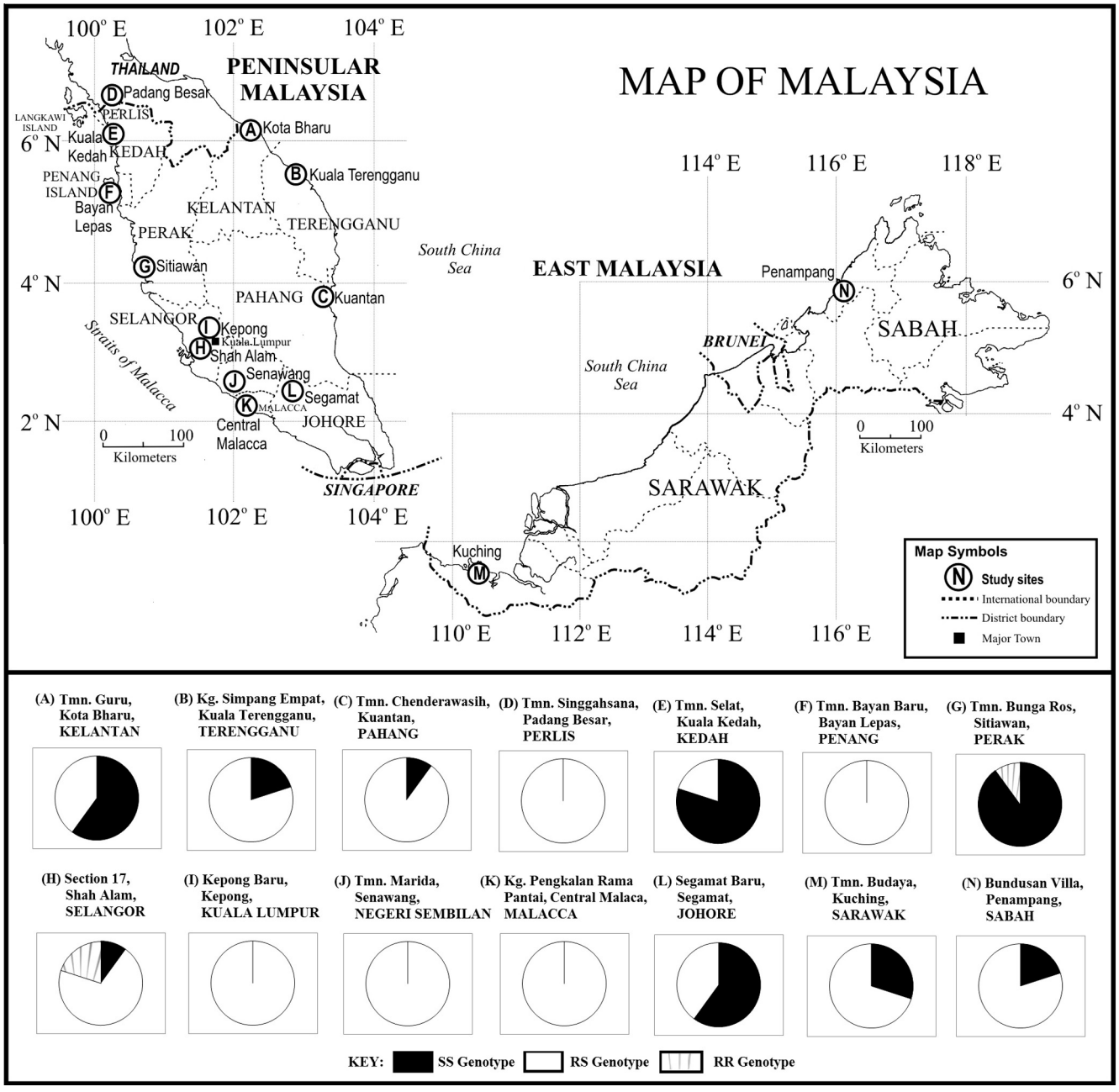

Fig. 1. Genotype distribution of *kdr* gene in *Culex quinquefasciatus* across all study sites in Malaysia. *Tmn. = Taman, Kg. = Kampung.

significantly ($P \leqslant 0.05$). The resistance *kdr* allele frequencies ranged from 0.1 to 0.55, with the highest being detected in *Cx. quinquefasciatus* population from Selangor (Table 1).

The results of DNA sequencing of 40 individual samples revealed only the presence of the 1014F mutation, while no other mutations were detected. Of these 40 individual samples, 24 were assigned as SS genotype, 13 as RS genotype and three as RR genotype. However, the results of DNA sequencing were not in complete agreement with AS-PCR method (Table 2). Of 40 samples, three individuals were assigned as SS genotype, but not RS genotype, which was contrasted with the AS-PCR results.

4. Discussion

The distribution of 1014 mutation(s) in *Cx. quinquefasciatus*, at varying frequencies has been reported worldwide [18,22–25]. In the current study, the classical knockdown resistance, 1014F mutation at varying frequencies was detected from all populations, while the 1014S mutation and other mutations reported previously were not detected in Malaysian *Cx. quinquefasciatus*. It has been documented that mosquitoes with 1014F mutation contributed high levels of resistance against both DDT and pyrethroids, while the 1014S mutation contributed high levels of resistance against DDT but low levels of resistance against pyrethroids [17]. Based on our previous report, the Malaysian *Cx. quinquefasciatus* populations displayed high levels of resistance against DDT but relatively low levels of resistance (or susceptible) against permethrin [15]. We propose that the widespread 1014F mutation occurred in Malaysian *Cx. quinquefasciatus* has resulted in the development of high DDT resistance. Likewise, a recent study also indicated that Indian *Cx. quinquefasciatus* with 1014F mutation demonstrated high DDT resistance but was susceptible against deltamethrin

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Table 1
Genotypes and frequency of *kdr* alleles in Malaysian *Culex quinquefasciatus*.

Localities		Genotype			Allele Frequency		HW
	n	SS	RS	RR	S	R	(*P*-value)*
Kelantan	10	6	4	0	0.80	0.20	1.00
Terengganu	10	2	8	0	0.60	0.40	0.17
Pahang	10	1	9	0	0.55	0.45	0.05
Perlis	10	0	10	0	0.50	0.50	0.01
Kedah	10	8	2	0	0.90	0.10	1.00
Penang	10	0	10	0	0.50	0.50	0.01
Perak	10	9	0	1	0.90	0.10	0.05
Selangor	10	1	7	2	0.45	0.55	0.52
Kuala Lumpur	10	0	10	0	0.50	0.50	0.01
Negeri Sembilan	10	0	10	0	0.50	0.50	0.01
Malacca	10	0	10	0	0.50	0.50	0.01
Johore	10	6	4	0	0.80	0.20	1.00
Sarawak	10	3	7	0	0.65	0.35	0.22
Sabah	10	2	8	0	0.60	0.40	0.17
Total	140	38	99	3	0.63	0.37	0.00

HW = Hardy–Weinberg test.
* The exact probability for rejecting Hardy–Weinberg equilibrium.

Table 2
kdr Genotypes detected by both AS-PCR and sequencing methods.

N	AS-PCR			Sequencing		
	TTA (SS)	TTA/T (RS)	TTT (RR)	TTA (SS)	TTA/T (RS)	TTT (RR)
40	21	16	3	24	13	3

[23]. However, this study does not exclude the involvement of metabolic mechanisms which can occur in the same populations, as observed by Djouaka et al. [26].

The present study reported the highest frequency of resistance *kdr* allele in Selangor population. One plausible explanation for this incidence could be permethrin and DDT resistance phenotypes evolved in this population, where the highest resistance ratio (3.81 folds) and low mortality rate (6.67%) were observed in our previous larval and adult bioassays, respectively [15]. In fact, a large number of dengue and chikungunya cases from the residential areas in Selangor have been persistently reported to the Ministry of Health, Malaysia. To control the outbreak of dengue and chikungunya fevers, permethrin fogging has been the preferred option since 1996 [13]. Consequently, the intense permethrin fogging activities for dengue vectors control has also exerted selective pressure on *Cx. quinquefasciatus* in these dengue and chikungunya endemic areas.

The findings of this study also indicated that RS genotype of 1014F mutation was the most predominant genotype and was well dispersed across majority of the study sites. A RR genotype was detected from two of 14 locations (i.e., Perak and Selangor). It has been reported that the absence of RR genotype in a population might alter the metabolic and developmental processes and consequently reduce its fitness-enhancing traits [27]. A previous study showed that high fitness cost has contributed to the rapid declination of the RR genotype after a few generations of insecticide-free conditions [28]. In the present study, it was observed that there was an excess of RS genotype recorded in most of the populations (i.e., Perlis, Penang, Kuala Lumpur, Negeri Sembilan and Malacca), probably due to the elimination of RR genotype in fitness cost evolution.

Attempts to determine the relationship between the frequency of *kdr* resistance allele with the insecticide susceptibility status in both larval and adult stages were made [15], but no association was found in either stage with regards to DDT and permethrin. Previous studies elsewhere have reported different relationship between the frequencies of the *kdr* resistant allele and the DDT and pyrethroids resistance phenotype. The insecticide resistance phenotype in several species of mosquito, house flies as well as the cockroach has been found to be correlated with the frequencies of *kdr* resistant allele [18,22,29–31]. Inversely, a number of studies also reported that no association was found between the *kdr* mutation and insecticide resistance phenotype in other insect species [23,32–34]. Given the lack of this association, it is possible that other detoxification mechanisms such as glutathione S-transferases and P450 monooxygenases could also be involved in DDT and pyrethroids resistance, respectively [4].

There have been many arguments about the accuracy of both PCR and sequencing methods for the detection of heterozygosity in an individual sample [35]. In the present study, we found that the results of DNA sequencing were not in agreement with the AS-PCR method. Similarly, previous studies also reported the incongruence results in both sequencing and AS-PCR methods [23,35].

We acknowledge that the estimation of single nucleotide polymorphism allele frequency could not be conclusively identified in the present study due to limited sample size. Therefore, for future study, additional sampling efforts with increased sample size from wider biogeographical areas should be carried out to provide a better understanding on the course of evolution in Malaysian *Cx. quinquefasciatus*. Nevertheless, the present study has demonstrated the first appearance of this widespread 1014F allele in Malaysian *Cx. quinquefasciatus* and documented the first field-evolved instance of knockdown resistance in insect species in Malaysia. This alarming case in the history of knockdown resistance development would pose a great challenge to both local authorities and researchers in the advancement of vector control management. It is possible that more than one resistance mechanism could confer DDT and permethrin resistance in these populations. Hence, the biochemical characterization of metabolic mechanism is currently in progress to unravel the actual mechanism(s) that contribute to the evolution of insecticide resistance.

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Research Article

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First molecular genotyping of insensitive acetylcholinesterase associated with malathion resistance in *Culex quinquefasciatus* Say populations in Malaysia

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Abstract

BACKGROUND: Given that there is limited available information on the insensitive acetylcholinesterase in insect species in Malaysia, the present study aims to detect the presence of G119S mutation in the acetylcholinesterase gene of *Culex quinquefasciatus* from 14 residential areas across 13 states and a federal territory in Malaysia.

RESULTS: The *ace-1* sequence and PCR-RFLP test revealed the presence of glycine–serine *ace-1* mutation in the wild populations of *Cx. quinquefasciatus*. Both direct sequencing and PCR-RFLP methods demonstrated similar results and revealed the presence of a heterozygous genotype at a very low frequency (18 out of 140 individuals), while a homozygous resistant genotype was not detected across any study site in Malaysia. In addition, statistical analysis also revealed that malathion resistance is associated with the frequency of *ace-1*^{R} in *Cx. quinquefasciatus* populations.

CONCLUSION: This study has demonstrated the first field-evolved instance of G119S mutation in Malaysian populations. Molecular identification of insensitive acetylcholinesterase provides significant insights into the evolution and adaptation of the Malaysian *Cx. quinquefasciatus* populations.

Keywords: G119S mutation; *ace-1*^{R}; propoxur; malathion; *Culex quinquefasciatus*; Malaysia

1 INTRODUCTION

Extensive use and overreliance on insecticides for vector-borne disease control have contributed to insecticide resistance development in the target species.[1] In Malaysia, susceptibility of mosquitoes against various insecticides has been studied extensively and described by various approaches such as WHO larval and adult bioassays,[2–8] enzyme microassays[9–12] and protein electrophoresis.[12] However, so far nothing has been reported pertaining to insecticide resistance gene detection at the molecular level. There is a dearth of evidence of insecticide resistance in Malaysian mosquitoes on a molecular basis.

Culex quinquefasciatus is one of the most common mosquitoes in residential areas in Malaysia.[13] Its significance as a vector of urban bancroftian filariasis has been documented in this region.[14] Specifically, insecticide resistance in the Malaysian *Cx. quinquefasciatus* has been well observed. Over the years, insecticide resistance towards carbamates and organophosphates in Malaysian *Cx. quinquefasciatus* has been reported.[4,6–9,11] Indeed, an elevated level of esterase activity has been identified to play a key role in organophosphate and carbamate resistance development in the mosquito.[9,11] Conversely, numerous studies have also reported that mutation at the acetylcholinesterase target site (G119S) is the main factor conferring resistance in organophosphates and carbamates.[15] However, in Malaysia, previous published studies have focused mainly on biochemical characterisation of the metabolic-based mechanism,[9–11] and there is a lack of evidence on insecticide resistance conferred by target-site insensitivity in this mosquito species.

Based on a previous report by the present authors, Malaysian *Cx. quinquefasciatus* populations have developed a wide spectrum of insecticide resistance towards propoxur and malathion, as demonstrated by WHO larval and adult bioassays. In addition,

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statistical analysis also revealed the presence of cross-resistance between propoxur and malathion.[8] Hence, this study was carried out to obtain further confirmation of incidences of cross-resistance between propoxur and malathion that were detected in the previous study, thereby attempting to investigate the prevalence of the *ace-1*^{R} mutation in *Cx. quinquefasciatus* populations from 11 states and a federal territory (Kuala Lumpur) in Peninsular Malaysia, as well as two states in East Malaysia that were separated by the South China Sea. In addition to providing a better understanding of the evolutionary relationship in this mosquito species, the information gathered from this study could improve the knowledge of vector control and management in Malaysia.

2 MATERIALS AND METHODS

2.1 Ethical notes

This research was regulated by the Medical Review and Ethics Committee (MREC), the Ministry of Health, Malaysia. No specific permits were required for this study, which did not involve endangered or protected species.

2.2 Mosquito strains

Mosquito larvae were collected from stagnant water at 14 residential areas across 11 states and a federal territory (Kuala Lumpur) in Peninsular Malaysia and two states in East Malaysia (Table 1 and Fig. 1) by using a previously described dipping method.[16] Because there is no specific control programme for *Culex* spp. in Malaysia, the selection criteria for these study sites were based on the frequent reports of dengue cases and fogging activities from these sites. Field-collected larvae were transported to the laboratory and reared to adulthood. Subsequently, the adult mosquitoes were identified according to the illustrated key.[17] In the present study, a total of 140 adults of *Cx. quinquefasciatus*, with ten individual mosquitoes representing each of the 14 study sites, were randomly selected.

2.3 WHO larval and adult bioassay

The larval bioassay was conducted according to the WHO[18] procedure. The bioassay was conducted in disposable paper cups of 300 mL capacity. The prepared stock solution of propoxur or malathion was added to 150 mL of deionised water. A total of 25 late third-instar or early fourth-instar larvae were introduced into the paper cups. Each insecticide consisted of five different concentrations with serial dilutions. After introducing larvae into paper cups, water was added to bring the final volume to 250 mL. Larval mortality was recorded after 24 h of continuous exposure.

The adult bioassay was conducted according to the WHO[19] procedure, with minor modifications. A total of 15 sucrose-fed female mosquitoes (3–5 days old) were exposed to propoxur 0.1% and malathion 5% WHO impregnated papers for 2 and 1 h respectively. The knockdown rate was recorded every minute for test insecticides, with their respective exposure. Survivability was recorded after 24 h of exposure.

2.4 DNA extraction

Prior to DNA extraction, abdomens were dissected from mosquito samples to avoid contamination. DNA was extracted from each specimen using an i-genomic CTB DNA extraction mini kit (iNtRON Biotechnology, Inc., Sungnam, Kyungki-Do, South Korea). All isolation steps were performed according to the instructions of the manufacturer.

2.5 Polymerase chain reaction (PCR)

The amplification of extracted genomic DNA was conducted using primers of *ace-1* from Cui *et al.*:[20] forward primer 5′-CGACTCGGACCCACTGGT-3′ and reverse primer 5′-GTTCTGATCAAACAGCCCCGC-3′. The amplification of the *ace-1* region was performed in a final volume of 50 μL containing 5 μL of 10$\times$ buffer, 2.5 mM of each dNTP, 10 pmol of each forward and reverse primer, 1.5 U of *Taq* polymerase (iNtRON Biotechnology, Inc., Sungnam, Kyungki-Do, South Korea) and 25–50 ng of genomic DNA of mosquito. PCR was carried out using a Bio-Rad MyCycler^{TM} thermal cycler (serial number 580BR 7200) (Bio-Rad Laboratories, Hercules, CA). The PCR conditions of *ace-1* included an initial denaturation of 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s (denaturation), 57 °C for 30 s (annealing), 72 °C for 1 min (extension) and a final extension at 72 °C for 5 min. A subset of 70 samples (including the 18 samples that exhibited the RS genotype by sequencing) was subjected to PCR-RFLP. The PCR fragments were digested with 1 μL of FastDigest *Alu* I (Thermo Fisher Scientific, Inc., Waltham, MA) for 15 min and fractionated on a 2% agarose gel prestained with SYBR Safe (Invitrogen, Carlsbad, CA).

2.6 DNA purification

The PCR products were purified with the MEGAquick-spin^{TM} PCR and agarose gel DNA extraction system (iNtRON Biotechnology, Inc., Sungnam, Kyungki-Do, South Korea). A total of 140 purified PCR products were sent to a commercial company for DNA sequencing in both directions. Samples were sequenced using BigDyeH Terminator v.3.1 sequencing kit and analysed on an ABI PRISMH 377 genetic analyser.

2.7 DNA sequence alignment

Sequencing data were analysed and edited using ChromasPro 1.5 (Technelysium Pty Ltd, Helensvale, Qld, Australia) and BioEdit 7.0.9.0.[21] The *ace-1* sequences were preliminarily aligned using the CLUSTAL X program[22] and subsequently aligned manually. Representative sequences of the *ace-1* gene of *Cx. quinquefasciatus* in this study were deposited in GenBank under the accession numbers JX575102 to JX575112.

2.8 Statistical analysis

WHO larval and adult bioassay data within the range 5–95% were subjected to probit analysis[23] using a computerised program.[24] Based on the LC_{50} and KT_{50} (50% lethal concentration and knockdown time) values, the resistance ratio (RR_{50}) was calculated by dividing values for the resistant strain by those for the susceptible strain.[25]

Heterozygous mutations were quantified on the basis of both forward and reverse sequences, where the heterozygous genotype (RS) exhibited double peaks in the mutation point, whereas the homozygous genotype (RR/SS) exhibited only one specific peak.[26] As for PCR-RFLP analysis, the two primers produced a fragment, which was undigested by *Alu*I for the SS genotype and cut into two fragments for the RR genotype. On the other hand, the RS genotype exhibited a combined pattern.[20,27] The frequencies of *ace-1*^{R} were determined by the Hardy–Weinberg equilibrium using GenePOP (v.3.4) software.[28]

The susceptibility status of larval (RR_{50}) and adult (survivability) bioassays was compared with the frequency of *ace-1*^{R} by Spearman rank-order correlation, using SPSS (v.18).

Table 1. Collection sites of *Culex quinquefasciatus* larvae across all states in Malaysia

Malaysia	Region	State	District	Collection site
Peninsular	East Coast	Kelantan	Kota Bharu	Taman Guru
		Terengganu	Kuala Terengganu	Kg. Simpang Empat
		Pahang	Kuantan	Taman Chenderawasih
	Northern	Perlis	Padang Besar	Taman Singgahsana
		Kedah	Kuala Kedah	Taman Selat
		Penang	Bayan Lepas	Taman Bayan Baru
		Perak	Sitiawan	Taman Bunga Ros
	Central	Selangor	Shah Alam	Section 17
		Kuala Lumpur	Kepong	Kepong Baru
	Southern	Negeri Sembilan	Senawang	Taman Marida
		Malacca	Central Malacca	Kg. Pengkalan Rama Pantai
		Johore	Segamat	Segamat Baru
East Malaysia	West	Sarawak	Kuching	RPR Batu Kawa
	East	Sabah	Penampang	Bundusan Villa

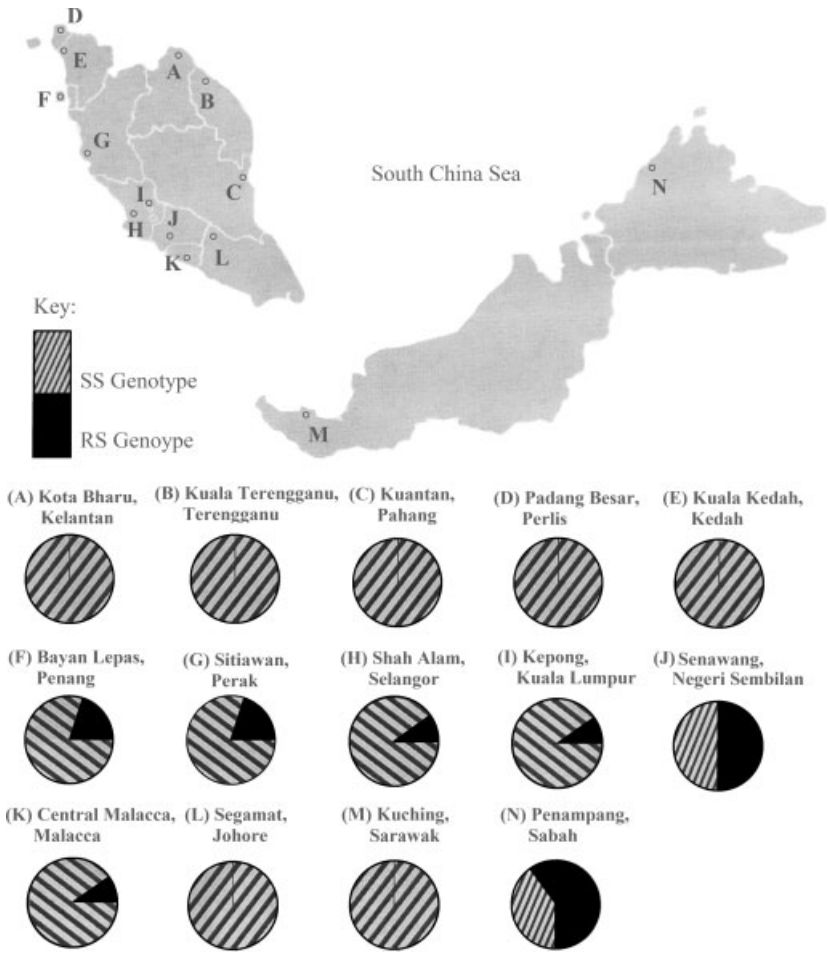

Figure 1. Genotype distribution of the *ace-1* gene in *Culex quinquefasciatus* across all study sites in Malaysia.

3 RESULTS

The larval bioassay demonstrated various resistance ratios across all study sites, 0.38–2.93-fold for propoxur and 0.36–13.88-fold for malathion. In the adult bioassay, the resistance ratios of propoxur ranged from 1.61 to 3.15, and those of malathion from 0.79 to 1.23. However, the resistance ratios of propoxur and malathion could not be determined by probit analysis (because of less than 5% knockdown in adults) from eight out of 14 and from seven out of 14 of the populations respectively, indicating that *Cx. quinquefasciatus* adults from these populations were highly resistant to both propoxur and malathion. Adult survivability recorded 24 h after the initial exposure period of propoxur and malathion ranged from 31.11 to 96.67 and from 0.00 to 100.00% respectively (Table 2).

Table 2. Susceptibility status of *Culex quinquefasciatus* against propoxur and malathion

	Propoxur					Malathion				
	Larval bioassay		Adult bioassay			Larval bioassay		Adult bioassay		
Strain	LC_{50}[a] (mg L^{-1})(95% CL)	RR_{50}[a]	KT_{50} (min)(95% CL)	RR_{50}	Survivability (%)	LC_{50}[a] (mg L^{-1})95% (CL)	RR_{50}[a]	KT_{50} (min)(95% CL)	RR_{50}	Survivability (%)
Susceptible	0.242 (0.234–0.251)	—	44.918 (43.642–46.134)	—	0.00±0.00	0.124 (0.114–0.135)	—	58.665 (57.464–60.319)	—	0.00±0.00
Kelantan	0.416 (0.382–0.454)	1.72	ND	—	96.67±3.34	0.045 (0.041–0.049)	0.36	56.653 (55.011–58.990)	0.97	3.33±3.34
Terengganu	0.092 (0.069–0.115)	0.38	79.643 (74.053–86.156)	1.77	45.00±5.00	0.057 (0.046–0.070)	0.46	46.632 (44.973–48.529)	0.79	0.00±0.00
Pahang	0.168 (0.146–0.189)	0.69	ND	—	80.00±5.77	0.168 (0.146–0.189)	1.35	52.822 (50.299–56.054)	0.90	0.00±0.00
Perlis	0.146 (0.116–0.177)	0.60	90.927 (87.977–94.248)	2.02	31.11±5.88	0.055 (0.051–0.060)	0.44	52.182 (50.724–53.939)	0.89	24.45±2.22
Kedah	0.489 (0.442–0.542)	2.02	141.512 (130.847–170.089)	3.15	93.33±3.85	0.864 (0.812–0.913)	6.97	70.862 (64.262–87.295)	1.21	28.89±5.88
Penang	0.708 (0.609–0.809)	2.93	ND	—	62.22±2.22	1.271 (1.216–1.322)	10.25	ND	—	64.44±5.88
Perak	0.175 (0.153–0.196)	0.72	ND	—	93.33±6.67	0.541 (0.514–0.568)	4.36	ND	—	90.00±3.33
Selangor	0.656 (0.580–0.725)	2.71	112.735 (109.257–116.973)	2.51	44.45±2.22	1.650 (1.592–1.714)	13.31	ND	—	100.00±0.00
Kuala Lumpur	0.623 (0.569–0.687)	2.57	ND	—	66.67±3.85	1.721 (1.651–1.806)	13.88	ND	—	88.89±5.88
Negeri Sembilan	0.456 (0.390–0.571)	1.88	ND	—	90.00±5.77	1.247 (1.203–1.300)	10.06	ND	—	93.33±6.67
Malacca	0.463 (0.413–0.514)	1.91	ND	—	84.45±2.22	1.309 (1.240–1.372)	10.57	ND	—	95.56±4.44
Johore	0.607 (0.547–0.675)	2.51	ND	—	77.78±2.22	1.009 (0.964–1.059)	8.14	ND	—	68.89±4.44
Sarawak	0.471 (0.426–0.517)	1.95	111.027 (107.677–115.038)	2.47	46.67±3.85	0.351 (0.306–0.396)	2.83	53.647 (52.265–55.318)	0.91	37.78±5.88
Sabah	0.384 (0.332–0.446)	1.59	72.211 (70.520–73.939)	1.61	37.78±4.45	0.700 (0.647–0.759)	5.65	71.958 (65.076–82.763)	1.23	44.45±2.22

[a] Details have been reproduced from a previous study by the present authors;[8] ND = not determined as the knockdown rate was less than 5%, indicating high resistance; CL = confidence limit; the adult survivability percentage was recorded 24 h after an initial exposure period of 2 h (propoxur) and 1 h (malathion).

Table 3. Genotypes and frequency of *ace-1* alleles in Malaysian *Culex quinquefasciatus*

Localities	*n*	Genotype SS	Genotype RS	Genotype RR	Allele frequency S	Allele frequency R	HW (*P*-value)[a]
Kelantan	10	10	0	0	1.00	0.00	0.00
Terengganu	10	10	0	0	1.00	0.00	0.00
Pahang	10	10	0	0	1.00	0.00	0.00
Perlis	10	10	0	0	1.00	0.00	0.00
Kedah	10	10	0	0	1.00	0.00	0.00
Penang	10	8	2	0	0.90	0.10	1.00
Perak	10	8	2	0	0.90	0.10	1.00
Selangor	10	9	1	0	0.95	0.05	1.00
Kuala Lumpur	10	9	1	0	0.95	0.05	1.00
Negeri Sembilan	10	5	5	0	0.75	0.25	1.00
Malacca	10	9	1	0	0.95	0.05	1.00
Johore	10	10	0	0	1.00	0.00	0.00
Sarawak	10	10	0	0	1.00	0.00	0.00
Sabah	10	4	6	0	0.70	0.30	0.48
Total	140	122	18	0	0.94	0.06	1.00

[a] HW = Hardy–Weinberg test. The exact probability for rejecting Hardy–Weinberg equilibrium.

Both sequencing and PCR-RFLP methods exhibited similar results and confirmed the presence of glycine–serine *ace-1* mutation in the wild population of *Cx. quinquefasciatus* (Fig. 1 and Table 3). Overall, the SS genotype was found in all 14 locations and was the most predominant, with 122 individuals from a total sample size of 140, followed by the RS genotype (18 individuals), while no RR genotype was detected across any state in Malaysia. Out of 14 populations, seven populations (Penang, Perak, Selangor, Kuala Lumpur, Negeri Sembilan, Malacca and Sabah) exhibited the G119S mutation, but only in the heterozygote state. Within these seven populations, the genotype frequencies were not significantly different from Hardy–Weinberg expectations at the 95% confidence level ($P > 0.05$). The *ace-1*^{R} allele was most widespread in *Cx. quinquefasciatus* from Sabah (*ace-1*^{R} allele frequency 0.30). Spearman rank-order correlation revealed that there was a significant correlation between the malathion resistance ratio and the frequency of the *ace-1*^{R} allele ($r = 0.543$, $P = 0.045$). In addition, at the adult stage, a significant correlation was also detected between the frequency of the *ace-1*^{R} allele and the malathion survivability rate ($r = 0.653$, $P = 0.011$) (Fig. 2). With regard to propoxur, no correlation was detected at either the larval or the adult stage.

4 DISCUSSION

The mutation involved in carbamate and organophosphate resistance that causes the replacement of a glycine (GGC) by a serine (AGC) at position 119 of the acetylcholinesterase gene has been documented in *Cx. pipiens* Linnaeus, *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus* Giles, *Anopheles nigerrimus* Giles, *An. atroparvus* van Thiel and *An. sacharovi* Favre since the 1980s.[15] More recently, this mutation has also been detected in *An. gambiae* and *An. albimanus*,[15,27] while a lack of evidence of this mutation occurs in *Aedes* mosquitoes.

With respect to *Cx. quinquefasciatus*, the distribution of G119S mutation at varying frequencies has been reported in this species.[20,29] In the present study, a very low frequency of G119S mutation was detected from seven populations, which suggests that the distribution of *ace-1*^{R} is a very recent event in Malaysia. A similar recent emergence of insensitive acetylcholinesterase has also been described in China.[20] However, it is important to point out that a wide spectrum of resistance levels against propoxur and malathion has been detected in *Cx. quinquefasciatus* populations that were collected concurrently from the same study sites.[8] As the frequency of G119S mutation was very low, it is possible that other detoxification mechanisms could be involved in insecticide resistance in this species, as reported by the local researchers in Malaysia.[9,11]

The findings of this study revealed that the SS genotype was the most predominant owing to its dispersal across all study sites, while a low frequency of the RS genotype was detected. The absence of the RR genotype in this study concurred with the findings of Alou *et al.*,[30] where the RR genotype was not detected in carbamate and organophosphate resistance in *An. gambiae* Giles s.s. populations from West Africa. Besides, a very low frequency of the RR genotype (one out of 100) has also been reported in field populations of *Cx. quinquefasciatus* from West Africa.[29]

Previous studies suggested that the absence of the RR genotype in a population might be due to the fitness cost of G119S mutation,[27,29,31] which involves alteration of metabolic and developmental processes that might reduce the fitness-enhancing traits.[32] Indeed, the insensitivity of the insecticide could reduce the normal function of enzyme in resistant individuals.[27,33] As a consequence of the high fitness cost, the frequency of the RR genotype could decline rapidly after a few generations in the absence of organophosphate or carbamate exposure.[34] In this study it was observed that there was an excess of RS genotype recorded in *Cx. quinquefasciatus* populations from Sabah, and this could be due to the elimination of the RR genotype in fitness cost evolution.

When comparing the relationship between the frequency of the *ace-1*^{R} allele and the status of WHO insecticide susceptibility bioassays at both larval and adult stages,[8] no correlation was found at either stage with regard to propoxur, indicating that other detoxification mechanisms might be involved in these populations. On the other hand, at the larval stage there was a significant correlation between the malathion resistance ratio and the frequency of the *ace-1*^{R} allele. Moreover, at the adult stage, a significant correlation was also detected between the frequency of the *ace-1*^{R} allele and the malathion survivability rate. These results suggested that G119S mutation is associated with malathion resistance in *Cx. quinquefasciatus* populations. However, other factors, such as the combination of several detoxification mechanisms, should be taken into consideration and would require further investigation.

It is true that the distribution of G119S mutation was detected at a very low frequency in the present study. Nevertheless, the results demonstrated that malathion resistance was associated with the evolution of G119S mutation and indicated the recent emergence of insensitive acetylcholinesterase in Malaysian *Cx. quinquefasciatus* populations. This first appearance of G119S mutation in the Malaysian mosquito is indeed a major problem for both local authorities and researchers in terms of monitoring the susceptibility status in the field and will pose a great challenge in insecticide resistance management. The importance of mosquito-borne diseases can be aggravated when a large proportion of RS

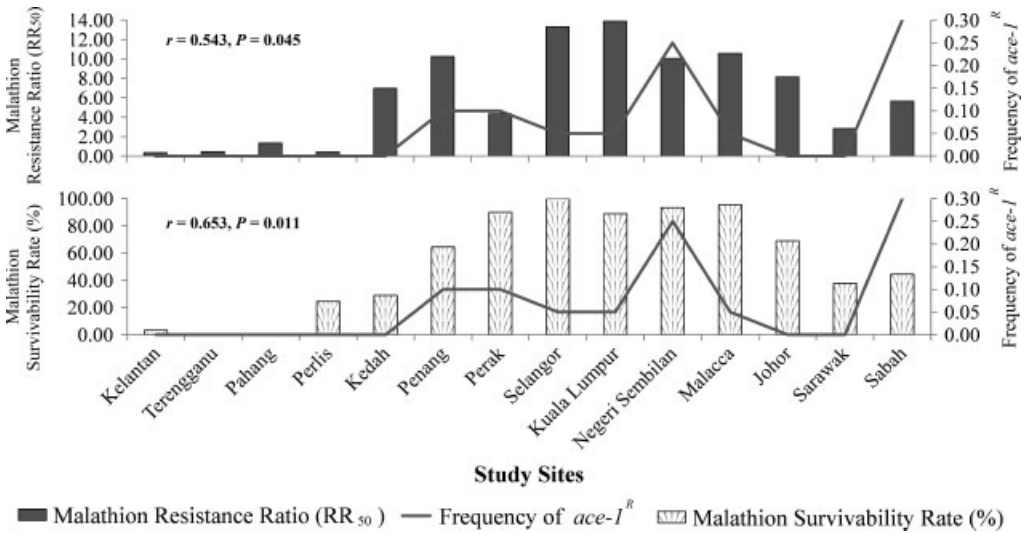

Figure 2. Spearman rank-order correlation between the frequency of *ace-1*^{R} and the malathion resistance ratio (larval stage) and malathion survivability (adult stage). Bars represent the malathion resistance ratio/malathion survivability; lines represent the frequency of *ace-1*.

genotype present in the wild populations exhibits the susceptible phenotype. For future study, it is recommended that a larger sample size from wider biogeographical areas be taken into consideration in the evaluation of susceptibility status against various classes of insecticides by conventional WHO bioassays supported by both molecular and biochemical evidence.

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